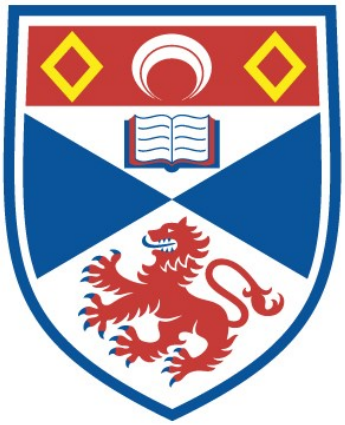
STRUCTURAL ASPECTS OF THE MEMBRANE AND ULTRASTRUCTURAL FEATURES OF SARCINA FLAVA AND SARCINA MORRHUAE

Marcus Ian Stuart Hunter

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1971

STRUCTURAL ASPECTS OF THE MEMBRANE AND ULTRASTRUCTURAL FEATURES OF SARCINA FLAVA AND SARCINA MORRHUAE

being a thesis presented by

Marcus Ian Stuart Hunter

to the University of St. Andrews in application for the degree of Doctor of Philosophy.

Th 5875

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr. D. Thirkell.

CERTIFICATE

I hereby certify that Marcus I.S. Hunter has spent nine terms engaged in research work under my direction, and that he has fulfilled the conditions of Ordinance No. 16, (St. Andrews) and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

[redacted]

ACADEMIC RECORD

I first matriculated at the University of St. Andrews in October 1964, and graduated with the degree of Bachelor of Science, First Class Honours in Biochemistry in June 1968. I matriculated as a research student in the Department of Biochemistry, University of St. Andrews, in October 1968.

ACKNOWLEDGEMENTS

I should like to express my gratitude to Dr. David Thirkell for his continued encouragement and advice throughout the course of this work. My thanks are also due to Mr. J. Crawford and Mr. W. Blyth for their able technical assistance.

STRUCTURAL ASPECTS OF THE MEMBRANE AND ULTRASTRUCTURAL FEATURES OF SARCINA FLAVA AND SARCINA MORRHUAE

C O N T E N T S

INTRODUCTION

A. THE ROLE OF CAROTENOIDS IN THE MEMBRANE OF GRAM-POSITIVE BACTERIA.

The observation that the carotenoids of a non-photosynthetic bacterium were entirely localised in the cytoplasmic membrane fraction was first made by Mathews and Sistrom, (1959a) for Sarcina lutea and this has subsequently been confirmed for several other species (Stephens and Starr, 1963; Salton and Ehtisham-ud-din, 1965a; Strang, 1968). This specific localisation may be significant when we consider the possible functions of such pigments.

Goodwin, (1959) observed that, of the Gram-positive bacteria whose natural environment involved contact with both light and oxygen, many synthesised carotenoid pigments. Carotenoids are also present in many microorganisms which are able to survive at physiological extremes, e.g. halophiles (Baxter, 1960), thermophiles (Baumann and Simmonds, 1969) and a radiation resistant species (Bamji and Krinsky, 1966).

It has been suggested that the pigments of S. lutea, because of their close association with the membrane-bound succinic oxidase system, may protect the enzymes and cytochromes of the respiratory chain by absorbing potentially harmful light energy (Mathews and Sistrom, 1959b). Protection by carotenoid pigments against the phenomenon of lethal photo-oxidation, (photo-dynamic killing) has been demonstrated when the bacteria were photosensitised with the dye Toluidine Blue (Mathews et al., 1959b, 1960; Mathews, 1963).

After normal wild-type cells of S. lutea and a pigment-less mutant of the same species were treated with Toluidine Blue, exposure of both types of cell to ultraviolet radiation resulted in the death of the cells lacking carotenoid pigment whilst most of the pigmented cells survived. This dye appeared to exert its lethal action at the level of the cytoplasmic membrane and its associated enzymes (Mathews, 1963). It is significant that no protection is afforded by the pigments of the same species after photosensitisation with Acridine Orange, whose prime lethal effect appears to be on the DNA and not on the membrane proteins of S. lutea (Roth, 1967). The possibility that carotenoid might be an integral structural component of the bacterial membrane is suggested by the observation that cells of S. lutea and Micrococcus lysodeikticus grown in the presence of diphenylamine, (which inhibits the synthesis of the more unsaturated carotenoids and carotenols), are more susceptible to lysis than normal cells. Furthermore, the membranes isolated from such cells appear to be less stable, as indicated by the leaching of membrane components into the supernatant during washing procedures (Salton and Freer, 1965; Salton and Ehtisham-ud-Din 1965a). However, Roth and Krinsky (1970) have suggested that in fact the carotenoid pigments do not play a significant role in stabilisation of the membrane since they were unable to demonstrate an increase in the fragility of either diphenylamine-grown or colourless mutant cells of S. lutea, nor of protoplasts prepared from these cells. They thus consider that the decrease membrane stability observed by previous workers for diphenyla-

mine-grown organisms is not a consequence of their depleted carotenoid content.

A novel suggestion for the function of the carotenoid in the membrane of Mycoplasma laidlawii is in the concerted transport of acetate out of, and glucose into the cell (Smith, 1969). Such a function presupposes that the dihydroxy-carotenol of this organism is located across the diameter of the micellar sub-units of the membrane. The two hydroxyls would be available for the covalent attachment of acetate and glucose, and rotation of the micelle through 180° would accomplish the transport process. Enzymatic fission of the O-acetate and O-glucose bonds by specific glycosidases would liberate these two compounds and allow the hydroxyls of the carotenol to become available for the repetition of the process.

B. NATURALLY OCCURRING CAROTENOID GLYCOSIDES AND CAROTENOID-PROTEIN COMPLEXES.

Many bacteria undoubtedly synthesise large amounts of free carotenoid pigment as the terminal product of their carotenoid biosynthetic pathway. This free carotenoid is easily extractable with organic solvents, after which the bacterial remnants are seen to be white, indicating that little or none of the pigment is tightly bound to e.g. protein (Thirkell, 1969; Weeks and Garner, 1967). In view of the evidence of Strang (1968) and others regarding the localisation of these pigments in the membrane, the carotenoid molecules must be orientated so that their hydrocarbon portions lie within the apolar region of this membrane, be it micellar or bi-layered in structure.

One such orientation has already been suggested by Smith (1969).

In many other organisms, however, the overall carotenoid biosynthetic pathway may well be directed towards the production of more polar compounds, of greater complexity, involving the attachment of carotenoid covalently to carbohydrate and/or protein. Until recently, it was thought that one such type of compound, carotenoid glycosides, was relatively rare but since 1967, the discovery of several of these in non-photosynthetic bacteria and in blue-green algae has indicated that carotenoid glycosides are widely distributed in nature. Some of the recently isolated carotenoid glycosides are listed in Table I.I. and examples of their structure are shown in Fig. I.I. It is of interest to note that, with one possible exception, in the glycosides of bacterial origin, D-glucose is the only carbohydrate moiety detected to date. Only two glycosides having C_{50} carotenoid aglycones have been reported so far.

The carotenoid glycosides are highly polar in comparison with the free pigments and as such are only sparingly soluble in even the most polar organic solvents. In those species which synthesise this type of compound, the glycosides represent a significant, if not a major proportion of the total carotenoid pigment (see Table I.I.) and it is evident that here the carotenoid glycosides are the terminal products of the biosynthetic sequence.

Water-soluble carotenoid-protein complexes are also abundant in nature, being especially common in invertebrates

TRIVIAL NAME	CAROTENOID (AGLYCONE)	SUGAR	% TOTAL PIGMENT	SOURCE ORGANISM	REFERENCE
None	3,3'-Dihydroxy-carotene (C_{40})	D-glucose (1)	?	M. laidlawii	Smith 1963
Phleixanthophyll	1',2'-Didhydro-1',2'-dihydroxy-ψ-carotene (C_{40})	D-glucose (1)	85	Mycobacterium phlei	Hertzberg & Jensen 1967
4-Keto-phleixanthophyll	1',2',-Dihydro-1',2'-dihydroxy-4-keto-ψ-carotene (C_{40})	D-glucose (1)		M. phlei	Hertzberg & Jensen 1967
P462	Hydroxychlorobactene (C_{40})	D-glucose (1)	15.7	Corynebacterium fascians	Prebble 1968
None	1,2-Dihydro-3,4-didehydro1-hydroxy-apo-8-lycopene (C_{40})	D-mannose (1)	64	Halophilic coccus, SE20-4	Aasen, Francis & Jensen 1969
Myxoxanthophyll	Myxol = 1',2'-Dihydro-3',4'-didehydro-3,1',2'-trihydroxy-ψ-carotene (C_{40})	L-rhamnose* (1)	?	Arthrospira sp.	Hertzberg & Jensen 1969a.
Oscillaxanthin	1,2,1',2'-Tetrahydro-1,2, 1',2'-tetrahydroxy-3,4,3',4'-tetradehydro-lycopene (C_{40})	L-rhamnose (2)	5	Arthrospira sp.	Hertzberg & Jensen 1969b
P496	Myxol (C_{40})	Unidentified O-methyl-methyl pentose (2)	9	Oscillatoria limosa	Francis, Hertzberg, Andersen & Jensen 1970
P476	Myxol (C_{40})	" " (1)	27	Oscillatoria limosa	Francis, Hertzberg, Andersen & Jensen 1970
P483	4-Keto-myxol (C_{40})	Unidentified methyl pentose (1)	1	Oscillatoria limosa	Francis, Hertzberg, Andersen & Jensen 1970
Corynexanthin	Decaprenoxanthin (C_{50})	D-glucose (1)	?	Corynebacterium sp. NCMB 8	Weeks & Andrewes 1970
None	Sarcinaxanthin (C_{50})	D-glucose (1)	20	S. lutea	Norgard, Francis, Jensen & Jensen 1970

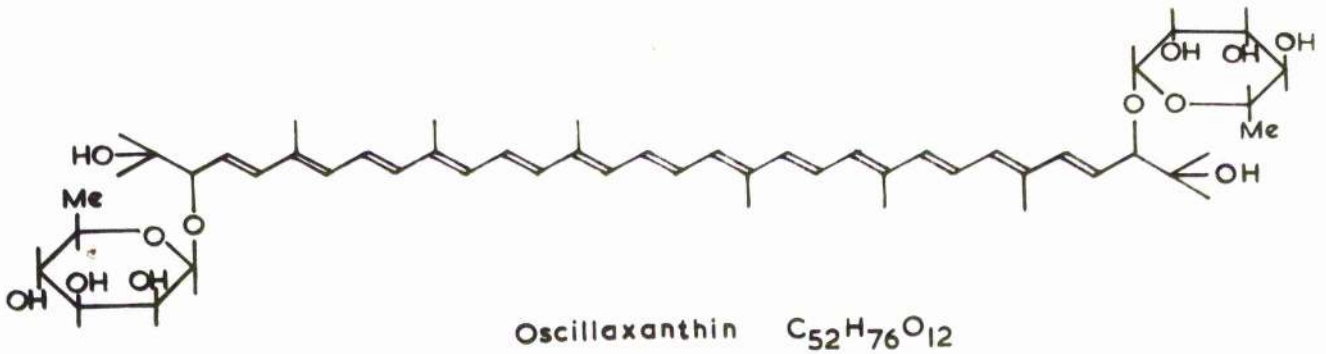
* Small quantities of an unidentified hexose were also detected in hydrolysates from this glycoside.

N.B. The configuration of the glycosidic linkage in the carotenoid glycosides is probably in common with most naturally occurring glycosides. Attempts at enzymic hydrolysis of the phlei-xanthophylls (Hertzberg et al. 1967) using both α- and β-glucosidases were unsuccessful in releasing free pigment, as demonstrated by chromatography of the reaction mixture.

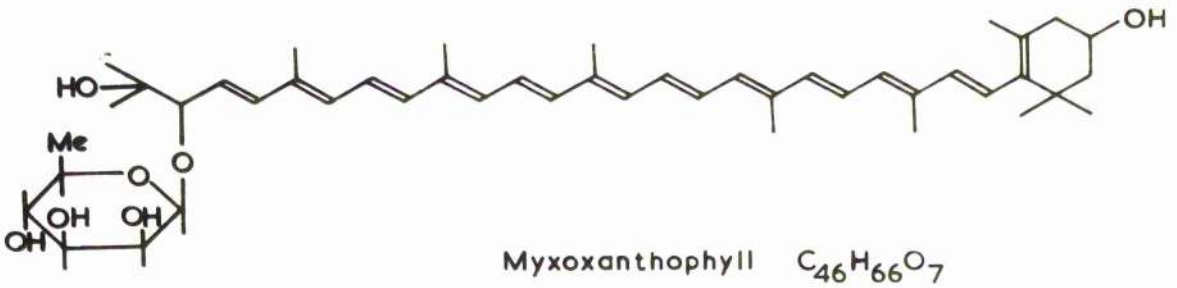
Figures in parentheses denote number of monosaccharide residues per carotenoid molecule.

TABLE I.I.

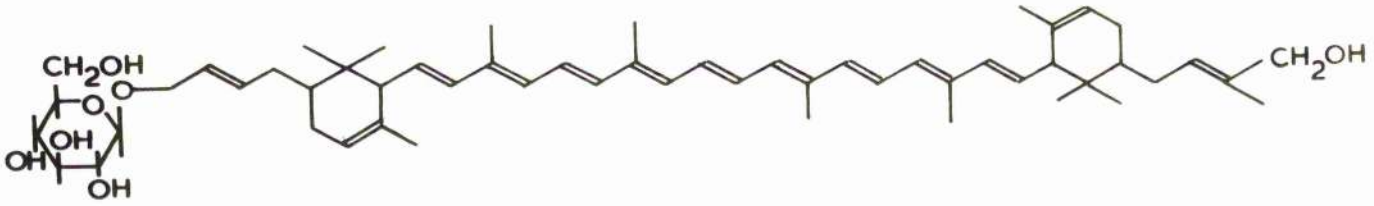
Recently Reported Naturally Occurring Carotenoid Glycosides



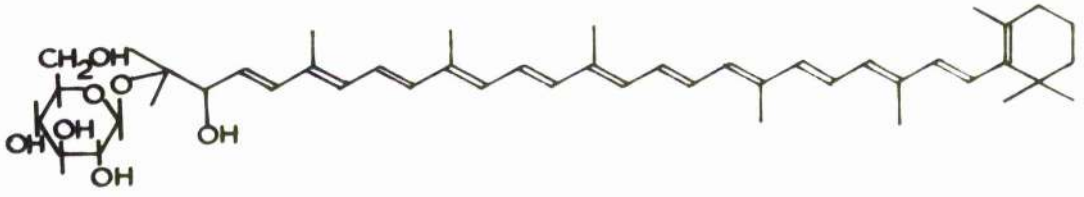
Oscillaxanthin $C_{52}H_{76}O_{12}$



Myxoxanthophyll $C_{46}H_{66}O_7$



Corynexanthin $C_{56}H_{82}O_7$



Phlei-xanthophyll $C_{46}H_{64}O_7$

FIG. I. I.

(Cheesman, Lee and Zagalsky, 1967). In this type of compound, the carotenoid moiety is easily released from the protein by treatment with protein denaturants e.g. acetone, but will recombine with the protein with the same stoichiometry as in the original material. Thus we have here a highly specific association between carotenoid and protein which, it has been demonstrated, confers mutual stability on both components. A further characteristic of these complexes from invertebrates is that the absorption maxima of the free and bound pigment differ considerably.

Readily extractable water-soluble carotenoproteins have also been isolated from algae (Haidak, Mathews and Sweeney, 1966), higher plants (Subbarayan and Cama, 1966) and the non-photosynthetic bacteria Corynebacterium michiganese (Saperstein and Starr, 1955) S. lutea (Mathews et al., 1959a), and a Clostridiu species (Mower H.F., 1970). These carotenoproteins are readily sedimentable by either high speed centrifugation or ammonium sulphate precipitation.

C. MEMBRANE SUB-UNITS SOLUBILISED BY THE USE OF SYNTHETIC DETERGENT AND ULTRASONICS.

In addition to the readily extractable carotenoproteins previously mentioned, synthetic detergents have been employed to extract carotenoid and protein-containing material from non-photosynthetic bacterial membranes (Razin, Morowitz & Terry 1965, Salton & Netschey 1965c, Salton 1967a,b, Butler, Smith & Grula 1967). These workers consider the detergent solubilised material to be a solution of identical sub-units on the basis of homogeneity on the analytical ultracentrifuge.

The use of detergents for obtaining soluble preparations from bacterial membranes is by no means confined to pigmented microorganisms (Bishop, Rutberg & Samuelsson 1967a, Mirsky 1969) but it is impossible to free the material so obtained from detergent so that all analytical procedures must be performed in the presence of detergent. The dangers inherent in this have been indicated by Smith, Koostra & Mayberry (1969), who showed that the sedimentation coefficient of detergent-solubilised material from M. laidlawii was dependant on the detergent concentration. These observations cast doubt on any attempt at comparison between the sizes of sub-units produced by this means and those produced by other purely mechanical procedures e.g. ultrasonics (see below).

Similar water-soluble pigmented membrane sub-units can be isolated after ultrasonication of bacterial membranes suspended in an aqueous buffer solution. In the case of M. lysodeikticus and S. lutea, these sub-units, yield a single, symmetrical peak on the ultracentrifuge, indicating that these sub-units are also homogeneous with respect to size (Salton et al., 1965). However, material prepared by ultrasonication of the membrane of M. laidlawii (Rottem & Razin 1966) exhibited an assymmetrical peak on ultracentrifugation suggesting some degree of heterogeneity of the sub-units.

D. THE CAROTENOID PIGMENTS OF SARCINA FLAVA.

Thirkell, Strang & Chapman (1967), showed that S. flava produces four major pigment fractions, as shown in Table I.II. None of the colourless carotene precursors constituting fraction 1

cochromatographed with the usual C_{40} carotenoid precursors e.g. phytoene, phytofluene and neurosporene, so that these authors suggest that the C_{50} skeleton is synthesised early in the overall biosynthetic pathway i.e. :-

Colourless C_{50} precursors -------> C_{50} carotene -------> C_{50} monohydroxy-carotenol -------> C_{50} dihydroxy-carotenol -------> C_{50} polar carotenoid.

In contrast, the C_{50} carotenoids from Flavobacterium dehydrogenans (see below) do appear to be synthesised from the normal C_{40} precursors. These precursors, along with lycopene and a C_{45} monohydroxy-carotenol can be isolated from this organism (Weeks, Andrewes, Brown & Weedon 1969) which suggests that the synthesis of the C_{50} structure occurs after the production of these coloured compounds with 40 and 45 carbons.

Thirkell & Strang (1967) have also shown that the carotenoid pigments of S. lutea and S. flava are chromatographically and spectrally similar. The same authors later showed (Strang & Thirkell 1969) that these pigments are also chromatographically similar to those produced by M. lysodeikticus and a Coryneform species (Strain 1032, Torry Research Station, Aberdeen). However, chromatographic and spectral evidence does not seem to be an adequate proof of structure since the C50 dihydroxy-carotenol from S. lutea (originally termed sarcinaxanthin by Takeda & Ohta 1941), is chromatographically homogeneous with, and possesses the same absorption maxima as the pigment p439 from F. dehydrogenans

FRACTION	TYPE OF CAROTENOID	NUMBER OF SUB-FRACTIONS	NATURE OF SUB-FRACTIONS	% OF TOTAL PIGMENT
1	Hydrocarbon	9	6 carotenes, 3 colourless precursors	5
2	Monohydroxy-C_{50}-carotenol	0	-	8
3	Dihydroxy-C_{50}-carotenol (Sarcinaxanthin ?)	0	-	26
4	Extremely polar	4	1 trans, 3 cisisomers	61

TABLE I. II.

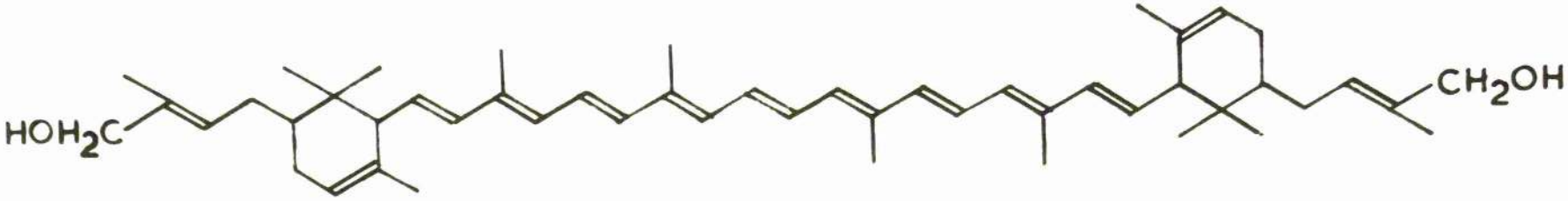
The Carotenoid Pigments from S. flava (after Thirkell, Strang & Chapman 1967).

(Jensen, Weeks, Strang & Thirkell 1967). Furthermore both pigments give the same highest mass number peak on mass spectrometry Nevertheless, subsequent studies of the fragmentation patterns of both pigments on mass spectrometry and by N.M.R. have revealed that P439, now termed decaprenoxanthin (Weeks et al. 1969) has the symmetrical structure shown in Fig. I. IIa) whereas sarcinaxanthin is an asymmetrical molecule whose tentative structure is either that shown in Fig. I. IIb) (Jensen 1970) or that shown in Fig. I. IIc) (Arpin, Francis, Jensen & Enzell 1970). It has now been shown that both F. dehydrogenans and a Coryneform species (NCMB8) synthesise decaprenoxanthin whilst S. lutea synthesises sarcinaxanthin.

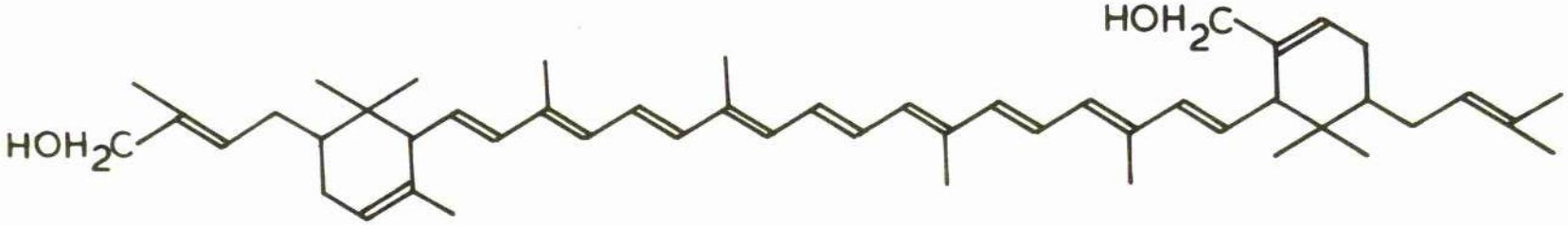
In view of the evidence of Thirkell et al., (1967) and the fact that S. flava is an extremely closely related, if not identical, species to S. lutea, sarcinaxanthin is probably the precursor of the polar carotenoid fraction (fraction 4) from S. flava, which was not further characterised by these workers.

E. THE CAROTENOID PIGMENTS OF SARCINA MORRHUAE AND OTHER RED, HALOPHILIC BACTERIA.

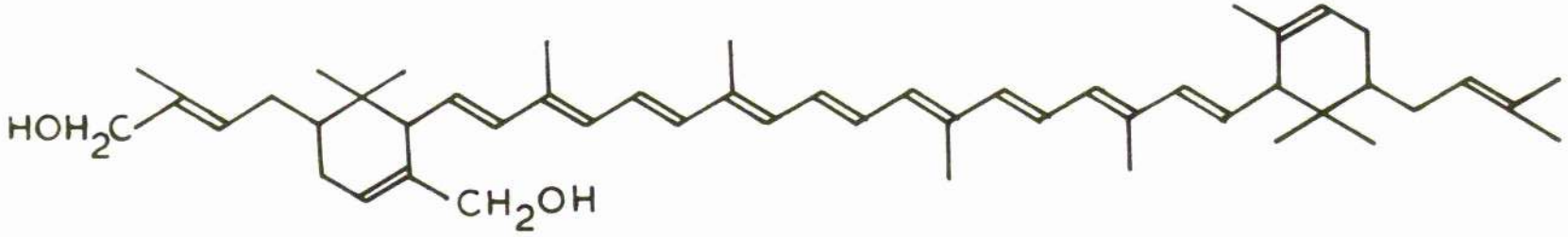
Many of the red pigmented halophiles, including Sarcina morrhuae, Sarcina littoralis and several species of the Halobacteri have all been shown to synthesise the same carotenoid pigments (Baxter 1960, Nandy & Sen 1967). Baxter (1960) demonstrated that the major pigment in these organisms was α-bacterioruberin, which had previously been reported as the major pigment in Halobacterium halobium (Petter 1931, Lederer 1938).



a) Decaprenoxanthin $C_{50}H_{72}O_2$ (Weeks et al., 1969)



b) Sarcinaxanthin $C_{50}H_{72}O_2$ (Jensen, 1970)



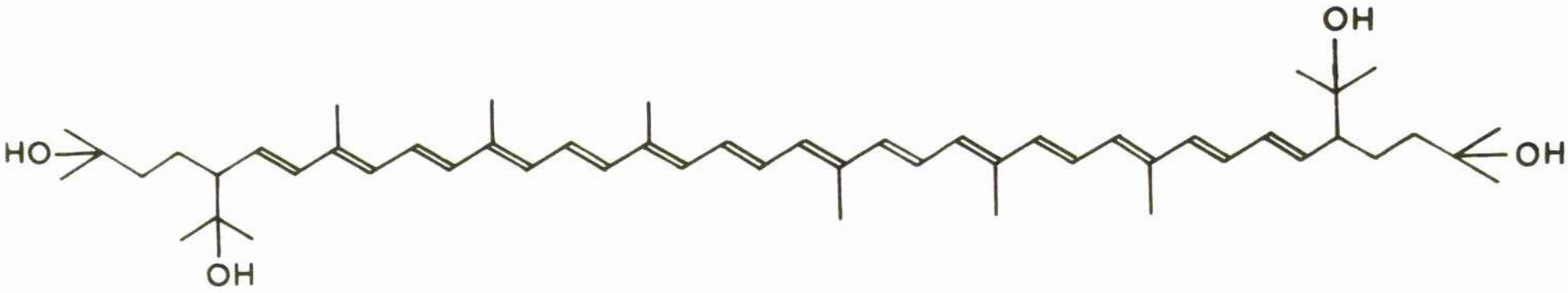
c) Sarcinaxanthin $C_{50}H_{72}O_2$ (Arpin et al., 1970)

FIG. I. II.

∝-bacterioruberin (or ∝-bacteriopurpurin) was until recently thought to be a C_{40} carotenoid but is now known to be a C_{50} compoun with the structure shown in Fig. I. III. (Kelly, Norgard & Jensen, 1970). It has been proposed (Kelly & Jensen, 1967) that the prefix be deleted since this is used to denote ∝-cyclogeranylidene end groups which are indisputably absent from the molecule.

Although the pigments of the red, halophilic organisms studied by Baxter (1960) are reported as being a mixture of eight fractions, with ∝-bacterioruberin as the major fraction, it has since been suggested (Nandy et al., 1967) that ∝-bacterioruberin only is synthesised and that all the minor fractions are derived from this compound by auto-oxidative and/or enzymic mechanisms, during its isolation. This would explain the non-reproducibility of Baxter's separations since the relative amounts of the pigment fractions would depend on the time interval between isolation and separation. The rate of transformation of ∝-bacterioruberin into these other derivatives was found to vary from species to species, being maximal in S. littoralis and minimal in H. halobium, of the organisms studied.

Baxter (1960) suggests that ∝-bacterioruberin is present in the cell as a pigment-protein complex which could be split using protein denaturing organic solvents, and whose absorption maxima differed significantly from those of the free pigment (c.f. Cheesman et al. 1967). Although Nandy et al. (1967) make no mention of the ease of pigment extraction from the bacteria which they examined, (S. littoralis, S. morrhuae, Sarcina sp. 63-R1 &



Bacterioruberin $C_{50}H_{76}O_4$ (Jensen et al., 1968)

FIG. I. III.

Halobacterium salinarium), Baxter (1960) in his work reports the impossibility of complete extraction of the carotenoids from S. littoralis cells, which do not lyse in hypotonic solution unlike those of the Halobacteria.

In this laboratory it was found that, when no further pigment could be extracted from cells of S. morrhuae, even after ultrasonication and treatment with 10% methanolic KOH, the bacterial remnants were still well pigmented. This residual pigment could be extracted as a reddish-orange aqueous solution.

F. THE CHEMICAL COMPOSITION OF GRAM-POSITIVE BACTERIAL MEMBRANES

a) Overall composition

As is the case for cellular membranes from higher forms of life, the bacterial membrane is composed predominantly of lipoprotein plus smaller amounts of carbohydrate and RNA.

All procedures for the isolation of membranes from Gram-positive microorganisms involve the removal of the cell wall using lysozyme (N-acetylmuramide glycanohydrolase, (E.C.3.2.1.17)), followed by protoplast lysis in hypotonic solution, centrifugation and washing procedures. The membrane fractions prepared in this manner represent the total membrane fraction from the bacterium i.e. the outer plasma membrane and any intracytoplasmic or mesosomal membranes which may be present.

The overall chemical compoisition of such membrane preparations undoubtedly varies according to the method employed for their isolation and purification, and the assay methods used

in the estimation of the various components. Salton (1967c) has shown that estimation of protein by the Folin-Lowry method gives a value which is 30% less than that obtained using the Biuret method. This sort of variation accounts for the widely differing chemical compositions frequently reported by different authors for membranes from identical species. Nevertheless, under identical conditions of preparation and assay, the chemical composition of bacterial membranes shows significant species variation (Salton et al., 1965b), as well as variation with the age of the culture (Salton et al., 1965b; Bishop, Rutberg & Samuelsson 1967b; Ward & Perkins 1968) and the composition of the growth medium (Salton et al., 1965b). The overall chemical composition of membranes from some Gram-positive bacteria is shown in Table I.III.

b) Carbohydrate composition

The qualitative carbohydrate composition of several Gram-positive bacterial membranes is shown in Table I. IV. Glucose is the most commonly occurring membrane carbohydrate and in several species is quantitatively the most important monosaccharide detectable (Salton et al., 1965b). Where ribose is present, this can be shown to be solely derived from the RNA component of the membrane. To date the only report of a pentose other than ribose in the membrane of a bacterium is that of Ghosh & Carroll (1968), who were able to detect arabinose and a trace of rhamnose in hydrolysates of membranes isolated from Listeria monocytogenes.

SOURCE ORGANISM	% PROTEIN	% LIPID	% CARBOHYDRATE	% RNA	% PHOSPHORUS	REFERENCE
B. megaterium	63	26	-	-	-	Mizushima, Ishida & Miura 1966
B. megaterium	70	25	-	-	-	Yudkin 1966
B. licheniformis	75	28	-	0.8	-	Salton et al. 1965b
B. subtilis	62	16	-	22	-	Bishop et al. 1967b
L. monocytogenes	55-60	30-35	1.3-2.3	1.5	3.5-4.0	Ghosh et al. 1968
M. lysodeikticus	52	28	15-20	-	-	Gilby, Few & McQuillen 1958
M. lysodeikticus	63-75	14.6-29.5	-	1.4-2.7	0.5-0.9	Salton et al. 1965b
S. lutea	39.8	28.9	9.6	1.2	-	Brown 1961
S. lutea	53-61	20.0-26.7	-	3.3-7.5	0.6	Salton et al. 1965b
S. aureus (protoplast)	66	25	0.89	2.74	1.06	Ward et al. 1968
S. aureus (L-form)	59.1	29.7	2.42	3.04	0.82	Ward et al. 1968
S. faecalis	49-55	28	-	-	-	Shockman, Kolb, Bakay Conover & Toennies 1963
S. pyogenes	68	15.3	-	-	-	Cohen & Panos 1966

TABLE I. III.

The Chemical Composition of the Membranes from Some Gram-positive Bacteria

SOURCE ORGANISM	TYPE OF MATERIAL	HEXOSE	HEXOSAMINE	PENTOSE	REFERENCE
B. licheniformis	Whole membrane	Glucose Galactose	-	-	Salton et al. 1965b
B. stearothermophilus	"	Glucose	-	-	Salton et al. 1965b
B. subtilis	Membrane lipid	Glucose	-	-	Bishop, et al. 1967b
S. lutea	Whole membrane	-	Yes, unidentified	-	Brown 1961
S. lutea	Whole membrane	Glucose Galactose Mannose	-	Ribose	Salton et al. 1965b
S. aureus	Whole membrane	Glucose	-	Ribose	Ward et al. 1968
S. aureus	Defatted membrane	-	-	Ribose	Ward et al. 1968
L. monocytogenes	Whole membrane	Glucose Galactose	Glucosamine	Ribose Arabinose Rhamnose	Ghosh et al. 1968
M. lysodeikticus	Defatted membrane	Mannose	Yes, unidentified	-	Gilby, et al. 1958
M. lysodeikticus	Whole membrane	Glucose Galactose Mannose	-	Ribose	Salton et al. 1965

TABLE I. IV.

Carbohydrate Composition of the Membranes from some Gram-positive Bacteria

None of the carbohydrate found in the membrane is present as free monosaccharide, but is released by acid hydrolysis from glycolipid, glycoprotein and in some cases polysaccharide (Gilby et al. 1958, MacFarlane 1964). The amount of carbohydrate present in membranes also varies markedly from species to species and from laboratory to laboratory for the same species. Thus Gilby et al. (1958) report a very high (20%) carbohydrate content for M. lysodeikticus membranes compared with more recent reports, but their isolation procedure did not eliminate the mannan which has been shown to be a membrane component under certain conditions (Salton 1967c). This polysaccharide constitutes 75% of the total membrane mannose, the remainder being predominantly in the form of a mannosyl diglyceride (MacFarlane, 1961).

In certain cases, specific monosaccharides can be assigned as components of the glycolipid of the membrane since a particular carbohydrate may be present in hydrolysates of whole membrane preparations but absent from hydrolysates of defatted membranes. In the case of S. aureus, glucose and ribose are demonstrable in whole membranes, but only ribose is detected in defatted preparations indicating that all the glucose is associated with the lipid of the membrane whilst, as we have said, the ribose is associated solely with the RNA. Thus for this species, little or no carbohydrate in the membrane is bound as either glycoprotein or polysaccharide (Ward et al., 1968).

The absence of a monosaccharide from membrane hydrolysates which is known to be present in the cell wall material, is a useful criterion of the purity of the membrane preparation (Gilby et al.

1958, Ghosh et al. 1968; Ward et al. 1968).

c) Amino Acid Composition

To the author's knowledge, amino acid analyses have only been reported for membrane proteins from:- M. lysodeikticus (Gilby et al. 1958, Grula, Butler, King & Smith 1967), S. aureus (Ward et al. 1968), B. megaterium (Yamagucai, Tsukakoshi, Tamura & Arima, 1967), S. faecalis (Shockman et al. 1963), and L. monocytogenes (Ghosh et al. 1968). These membrane proteins, in common with those from higher forms of life, are characterised by a high content of hydrophobic and acidic amino acid residues, and a low content of basic and sulphur-containing amino acids. This precludes extensive ionic binding between phospholipid and protein and the possibility of significant disulphide cross-linking in the membrane.

All the usual amino acids can be detected in most cases, as well as some unidentified (Grula et al. 1967) and some unusual components e.g. phosphoserine and glyceryl phosphoryl ethanolamine from L. monocytogenes (Ghosh et al. 1968).

Little work appears to have been done concerning the effect of alterations in culture conditions on the amino acid patterns of membrane proteins. Shockman et al. (1963), however, noted that membranes from threonine depleted cells of S. faecalis showed an increased content of lysine and glutamic and aspartic acids, the remainder of the amino acids being reduced by 20-30%. It is also interesting that membranes from protoplasts of S. aureus contain 33-49% more alanine than those from L-forms of the same species

(Ward et al. 1968), although the significance of this difference has not yet been explained.

The absence of unusual amino acid components, known to be cell wall constituents e.g. diaminopimelic acid, from membrane preparations is again a useful indication of the efficiency of the isolation procedure (Gilby et al. 1958).

d) Fatty Acid Composition

The number of reports concerning the fatty acid composition of whole bacteria are too numerous to mention here and there are two excellent reviews concerned with this subject (O'Leary 1962, Kates 1964). Only a few workers have concerned themselves with the fatty acid composition of isolated bacterial membranes (Cho & Salton 1966, Bishop et al. 1967, Yamaguchi et al. 1967, Ward et al. 1968) but since there is good evidence that, for some Gram-positive species, most, if not all the lipid is localised in the membrane (Vorbeck & Marinetti 1965), it may be reasonable to assume that the fatty acid profile obtained from whole cells in these cases will be effectively an expression of the membrane profile. Further support for this supposition comes from the observation by the same workers that where a small amount of lipid was found in the soluble cytoplasmic fraction, this lipid was of the same composition as that from the membrane.

The predominant fatty acid of several aerobic Gram-positive bacteria, including S. lutea and M. lysodeikticus (Cho et al. 1966) and many of the Bacillus species (Kaneda 1967), is a saturated, branched-chain C_{15} compound. Other branched-chain acids are also common in these species, and their content of unsaturated acids is

low in comparison with Gram-negative species.

Fatty acid composition has been suggested as a criterion for bacterial classification by several workers (Moss, Dowell, Lewis & Schekter 1967, Lewis, Weaver & Hollis 1968, Moss, Dowell, Farshtchi, Raines & Cherry 1969, Kaneda 1967), although variations in the composition of the growth medium (Kaneda 1966, Tornabene, Bennett & Oro 1967, Edmonds & Cooney 1968, Albro & Dittmer 1969), availability of oxygen (White & Frerman 1968), temperature (Marr & Ingraham 1962, Kates & Hagen 1964, Daron 1969, Ray, White and Brock 1971), and the age of the culture (Knivett & Cullen 1967, Kaneda 1966, Daron 1969), have all been shown to produce marked changes in the fatty acid profile from whole bacteria. Tornabene *et al.* (1967), however, found no significant alteration in the fatty acid pattern for cultures of *S. lutea* grown for 24, 48 and 96 hours.

G. ULTRASTRUCTURE AND CELL DIVISION OF GRAM-POSITIVE BACTERIA

Compared with the wealth of material published on other Gram-positive bacteria e.g. the *Bacilli*, ultrastructural studies on the *Micrococci* and *Sarcinae* are few. Those species which have been investigated are *S. lutea* (Chapman 1960, Cherny 1967), *S. ventriculi* and *S. maxima* (Holt & Canale-Parola 1967), *S. morrhuae* (Brown & Cho 1970), *M. lysodeikticus* (Friedberg & Avigad 1968) and *M. radiodurans* (Thornley, Horne & Glauert 1965).

Chapman (1960) reported that *S. lutea* cells were bounded by a single, thick layer, intermediate in appearance between a cell wall and a cytoplasmic membrane. This layer grew centripetally inwards to partition the cell during the division process.

A later investigation of the ultrastructure of S. lutea (Cherny 1967) revealed the existence of both the typical thick amorphous cell wall, and a tripartite cell membrane, characteristic of the majority of the Gram-positive microorganisms studied. In addition, extensive intracytoplasmic membrane structures or mesosomes were present as well as the large polymetaphosphate granules which have since been demonstrated in the closely related species M. lysodeikticus (Friedberg et al. 1968).

The behaviour and function of the bacterial cytoplasmic membrane during cell division was first described by Chapman (1959) although subsequent authors have assigned roles to both the cytoplasmic membrane and to mesosomes in this process. Chapman considers that bacterial cell division may be of two types : cell wall septation (Chapman & Hillier 1953, Chapman 1960) or cell membrane septation (Chapman 1959). Although centripetal growth of a ring of material is common to both processes, the former is usually characterised by the association of mesosomes with the developing cell wall septum. In the latter process, cell membrane septation, a double membrane septum is formed, completely separating the two halves of the cell and this is then followed by the deposition of cell wall material on the membrane septum.

More recent work (Jacob, Ryter & Cuzin 1966, Ryter & Jacob 1967, Ryter 1967, Petitprez, Roos & Tacquet 1967, Suganuma 1968) has shown that for many species there is an intimate association between mesosomes and the bacterial DNA, and that mesosomes may well participate in nuclear segregation during cell division.

Little effort seems to have been concentrated on the actua mode of septum formation during cell division in Gram-positive bacteria although cell wall septation, accompanied by mesosomes in close proximity to the developing septa, is shown in B. cereus (Chapman et al. 1953), B. subtilis (Glauert & Hopwood 1961, Ryter & Jacob 1964, Cole, Popkin, Doylan & Mendelson 1970), S. maxima (Holt et al. 1967), M. phlei (Petitprez et al. 1967), S. aureus (Suganuma 1968), S. faecalis (Higgins & Shockman 1970), and Corynebacterium ovis (Hard 1969).

In their work on Diplococcus pneumoniae, Tomasz, Jamieson & Ottolenghi (1964), described the presence of membraneous filaments termed S-membranes, continuous with the cytoplasmic membrane, and extending terminally from developing septa in dividing cells. Mesosomes were seen on occasions in the same position as these S-membranes, but never simultaneously. As division in this organism is of the cell membrane septation type, here we have an exception to the general observation that cell membrane septation is characterised by the absence of mesosomes at the site of septation.

H. AIMS OF THIS THESIS

The work presented here was undertaken with a view to achieving the following objectives:-

1. Solubilisation, using synthetic detergent, of a carotenoid-containing membrane component from the membranes of *S. flava*, and the investigation of its properties, in an attempt to determine the way in which the pigment is incorporated into the general architecture of the membrane.

2. Further characterisation of the polar carotenoid fraction from *S. flava* as isolated by Thirkell *et al*. (1967) to determine the nature of the substituents and/or other molecules attached to the carotenoid which are responsible for its extreme polarity.

3. Elucidation of the composition and properties of a water soluble pigment fraction from *S. morrhuae*.

4. Determination of the effect of age of bacterial culture on the chemical composition of the total membrane fraction from *S. flava*.

5. Electron microscopic examination of *S. morrhuae* and *S. flava*, with particular emphasis on the mode of cell division in, and the effect of lysozyme treatment on, *S. flava*.

METHODS

A. MAINTENANCE AND LARGE SCALE CULTURE OF SARCINA FLAVA AND SARCINA MORRHUAE

1. Maintenance culture of S. flava

S. flava, (N.C.T.C. 7503), was grown on nutrient agar (Oxoid Ltd.), enriched with 1% glucose, at 37°. A single colony was transferred to a fresh agar plate every 5 days, and a new maintenance culture thus prepared by streak culture.

2. Large scale culture of S. flava

a) Cells were grown in a New Brunswick MF 114 Microferm bacterial fermenter at 37° in 12 litres of nutrient broth (Oxoid Ltd.), enriched with 1% glucose. The cultures were vigorously agitated throughout the fermentation and aerated at a rate of 12 l./min. Harvesting was by centrifugation at 1500 g. and the times for which different batches were grown is indicated at the appropriate points in this section.

b) Cells were grown (at Imperial College, London), in 400 l nutrient broth (Oxoid Ltd.) enriched with glucose as above. Vigorous agitation and an air flow rate of 400 l./min. were employed throughout. Two batches of cells were grown at Imperial College, one being harvested after 48 hours growth, the other being divided into two 200 l. portions, one of which was harvested after 24 hours, the other being allowed to continue growth for 57 hours before harvesting. In each case, harvesting was carried out on a Sharples continuous flow centrifuge, and the cells deep-frozen for transport.

The approximate yield of S. flava cells grown in liquid culture under these conditions is 7.5 g. (wet weight)/l.

3. The effect of light on the growth and pigmentation of S. flava

A series of universal bottles, each containing 20 mls. of nutrient broth (Oxoid Ltd.), enriched with 1% glucose were each inoculated with 0.5 ml. of a 24 hour culture of S. flava. Half of the bottles were made light proof using aluminium foil, the remainder being left uncovered. All were rotated on a roller-shaker at 34° with overhead illumination from a fluorescent strip light. At daily intervals for up to 7 days, aliquots were withdrawn sterilely from each bottle, suitably diluted where necessary, and monitored for bacterial numbers by turbimetric measurement at 610 nm. When peak numbers had been reached, the remaining cells were harvested by centrifugation, and the light and dark grown cells pooled separately. The carotenoid pigment was then extracted by ultrasonication in a known volume of methanol, and estimated using a theoretical extinction coefficient ($E^{1\%}_{1cm}$ = 3000) first used by Strang (1968) for the pigments from this organism. The amount of pigment present in the light and dark grown cells was related to the dry weight of the bacteria.

4. Culture of S. morrhuae

The extreme halophile, S. morrhuae, was grown on 2.5% agar No.3 (Oxoid Ltd.), to which was added : 25% NaCl; 1% $MgSO_4.7H_2O$; 0.02% $CaCl_2.6H_2O$; 0.5% KCl; 1% yeast extract (Oxoid Ltd.); and 0.5% tryptone (Oxoid Ltd.). (All percentages here are w/v). The agar plates were streak cultured with the microorganism and sealed polythene bags in order to minimise moisture loss and subsequent crystallisation of salt on the surface of the solid medium. The

plates were incubated at 34° for three weeks after which time the cells were harvested by careful scraping with a spatula.

B. PREPARATION OF MEMBRANES AND PROTOPLASTS FROM WHOLE CELLS OF S. FLAVA

1: Preparation of the total membrane fraction

Membranes were prepared from cells grown for 24, 48, 57, 72 and 91 hours, by the method of Salton et al. (1965b). Tris buffer was used throughout the preparation since these workers found that the use of phosphate buffer caused a greater degree of membrane instability. The method is depicted in the flow scheme shown in Fig. M.1. (overleaf). The purified membrane fraction thus prepared was either used immediately (as in Section C), or lyophilised and stored at -40° for later work (as in section F.).

2. Estimation of yield of membrane fraction

Membranes were prepared by the above method, from duplicate 500 mg. samples of lyophilised whole bacteria, harvested at 24, 57 and 91 hours. The weights of lyophilised purified membranes thus obtained were determined, and the yield of the total membrane fraction for each age of cell estimated.

3. Preparation of protoplasts

Protoplasts were prepared by the method of Baird-Parker and Woodroffe (1967), which is stated to be suitable for both B. megaterium and M. lysodeikticus. 100 ug./ml. of egg white lysozyme (Sigma Ltd.) were added to a suspension of cells (20 mg. wet weight/ml.) in a medium containing: 0.01 M Sorensen double phosphate buffer, pH 7.0; 0.005M $MgCl_2$; 0.01M NaCl and 0.75M sucrose. Despite the contention in the original method that

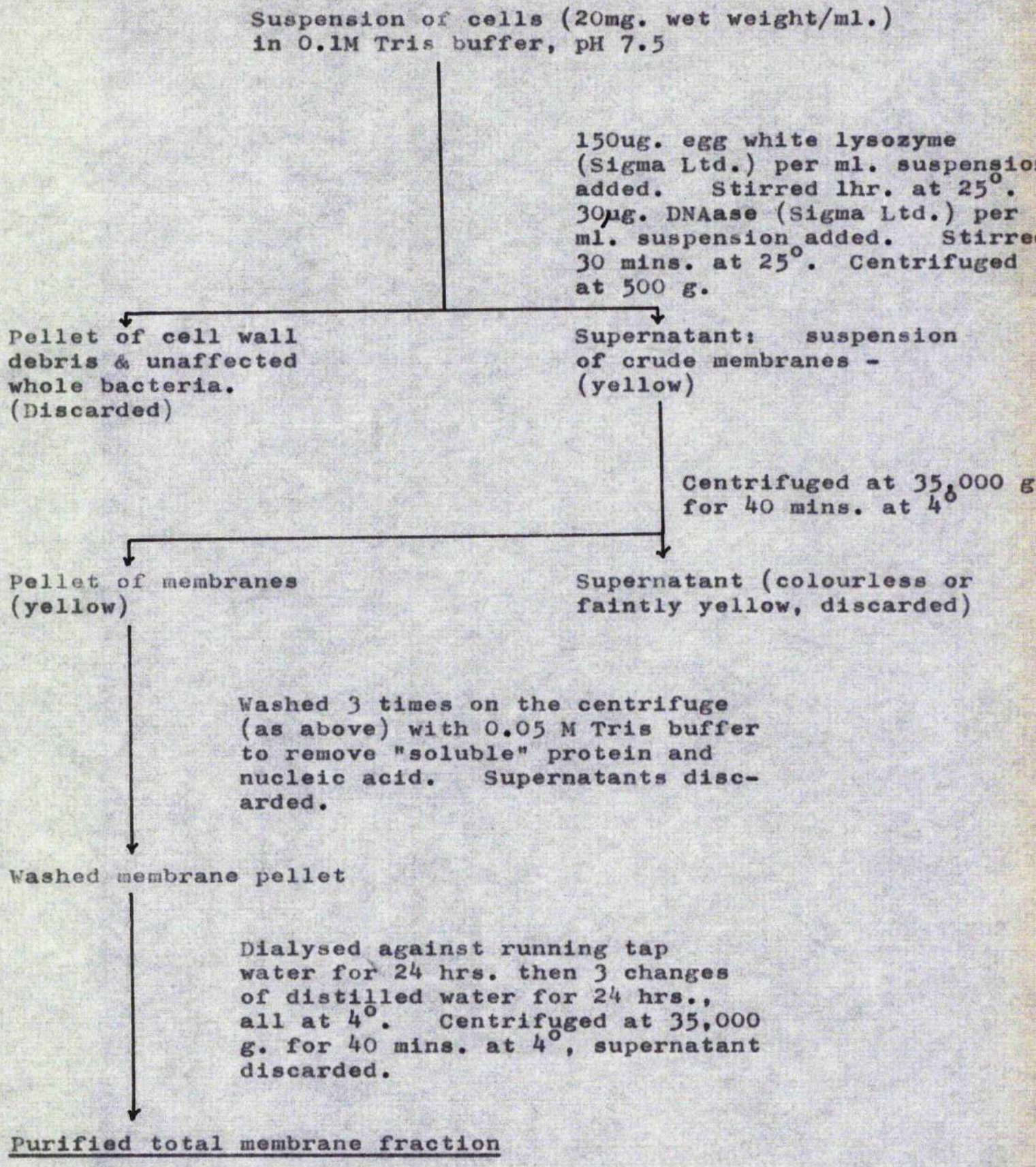


Fig. M. 1. - Method for the preparation of the total membrane fraction from S. flava (after Salton et al., 1965b).

protoplasting was complete after only 15-20 mins., for S. flava the suspension was incubated at 30° for 60 mins. The protoplasts were then collected as a pellet after centrifugation at 35,000 g. for 40 mins. and portions were fixed for electron microscopy as in Section G.

C. PREPARATION AND PROPERTIES OF A DETERGENT-SOLUBILISED CAROTENOID GLYCOPROTEIN FROM S. FLAVA MEMBRANES

1. Method of isolation and purification

Freshly prepared membranes from a 72 hour culture of S. flava were suspended in a 1% solution of either Lubrol L (I.C.I. Ltd.) or Crillet 1 (Croda Ltd.), in 0.1 M Tris/maleate buffer pH 7.2, to a final concentration of 50 mg. wet weight/ml. Both detergents are non-ionic in character, being derivatives of poly-oxyethylene. The membrane suspensions were shaken for 24 hrs. at 25° and the solubilised material decanted off from the residue after centrifugation at 100,000 g. for 30 mins. The extraction was then repeated until no more colour could be obtained in the supernatant (usually 3 times), after which the membrane remnants were still faintly yellow.

The efficiency of this solubilisation procedure was determined by the accurate weighing of the residue obtained after the extraction of a known weight of membranes.

In an attempt to remove associated detergent, the combined yellow supernatants were exhaustively dialysed against: a) running tap water for 24 hrs., and b) 3 changes of distilled water for 24 hrs. All dialysis was carried out at 4°.

The dialysed material was then made 30% (w/v) with respect to $(NH_4)_2SO_4$, which precipitated the yellow gelatinous carotenoid glycoprotein leaving a colourless supernatant after centrifugation at 100,000 g. for 30 mins. at 4^o. The yellow pellet was re-dissolved in distilled water, and the precipitation procedure repeated 3 times, after which the material was dialysed again as above to remove $(NH_4)_2SO_4$. The dry weight and ash content of the first solution prepared in this manner were determined in the usual way. The concentration of subsequent batches was determined by refractometry using a P.C.L. differential refractometer.

2. Attempted resolution of the purified detergent-solubilised materials

a) Electrophoresis in free buffer film

This technique was developed by Grassman and Hannig in Munich and the machine used was of the vertical flow type, manufactured by Bender and Holbein. Before introduction into the apparatus, aliquots of the material were exhaustively dialysed against a wide range of buffers of varying ionic strength and with pHs ranging from 3.9 to 8.6.

b) Disc-gel electrophoresis

Polyacrylamide gels were prepared as follows:-

Solution I : 48 mls. M KCH + 17.2 mls. glacial acetic acid + 4 mls. tetramethylethylenediamine (TEMED), made up to 100 mls. with distilled water.

Solution II : 30 gms. Cyanogum 41 (B.D.H. Ltd.) in 100 mls. distilled water.

Solution III : 0.004 gms. riboflavin in 100 mls. dist. H_2O

Gel solution : 1 vol. I + 2 vols. II + 4 vols. III + 1 vol. distilled water.

The gel solution was pipetted into the gel tubes to give the same height of gel in each tube, overlayered with a small amount of water, and allowed to polymerise (10-30 mins.).

The gels were placed in the apparatus (Shandon) with 0.005 M acetate buffer, pH 4.7 in both upper and lower chambers and prerun for 1 hr. at 4 m.a. per gel. Solutions of both types of detergent-solubilised material and of egg white lysozyme and bovine serum albumin were prepared to give a final concentration of about 1 mg./ml. in each case. These sample and standard solutions were then made 5% (w/v) with respect to sucrose (to minimise diffusion effects on application) and carefully applied to the top of the gels with a syringe to give a layer of approximately 1 mm. thickness. Electrophoresis was then performed at a current of 4 m.a. per gel and constant voltage for 3 hours.

The gels were carefully removed from their tubes and stained in 1% amide black (1 gm. in 100 mls. 7% glacial acetic acid) for 2 hours. Destaining of the gels was accomplished by : a) washing with distilled water; b) soaking in 7% glacial acetic acid for 1 hour; c) electrolytic destaining in 7% glacial acetic acid at 150 volts for 1 hour, after which the bands were clearly visible.

c) Sephadex gel-filtration

Samples of both types of detergent solubilised material were concentrated by the forced evaporation of water from the outside of dialysis sacking containing the solutions, using a stream

of cold air from an industrial blower. 2 mls. of concentrated material were applied to glass columns (26 X 1.5 cms.) containing G100 and G200 Sephadex respectively. The columns were eluted with 0.1% (w/v) NaCl and the 3 ml. fractions which were collected were monitored for absorbance at 444 nm. (carotenoid) and by the Folin-Lowry (Lowry, Rosebrough, Farr & Randall, 1951) method (protein).

d) Ultracentrifugation

This was carried out using a Beckman Spinco model E analytical ultracentrifuge. The sedimentation patterns of solutions of both types of material in distilled water were examined, using Schlieren optics, for up to 2 hours following the attainment of maximum rotor speed (59,780 r.p.m.). The possibility that the material might be split into sub-units was investigated by examining the sedimentation behaviour of solutions of the Lubrol L solubilised material containing : a) 4 M urea; b) 4 M urea + 1% (v/v) thioglycollic acid.

3. Properties of the detergent-solubilised carotenoid glycoprotein prepared using Lubrol L

a) Absorption spectrum

The absorption spectrum was determined for the range 200-700 nm. using a Pye Unicam S.P. 800 instrument. This was repeated, using a heated cell carriage, at 5° increments from 15°-100°, and after prolonged boiling of the material. The spectra of solutions titrated to pH 4.0, 7.0 and 10.0 were also read.

b) Optical rotation

Optical rotation was determined using a Hilger and Watt

polarimeter, with a water-jacketed polarimeter tube, over the temperature range 18^{o}-78^{o}.

c) Amino acid analysis

A solution containing approximately 100 mg. of the material was taken to dryness and redissolved in 10 ml. 6 N HCl in a hydrolysis tube. This was then sealed and hydrolysis allowed to proceed for 24 hrs. at 110^{o}. The hydrolysate was filtered to remove humin, which was then washed twice with distilled water to ensure quantitative recovery of the amino acids. The filtrate was then taken to dryness on a rotary evaporator, followed by the addition of a small volume of distilled water and the process was repeated until the HCl had been removed. The amino acid composition of the hydrolysate was then determined using a Technicon amino acid analyser, with nor-leucine as an internal standard.

d) Carbohydrate detection and chromatography

In order to determine whether or not carbohydrate was present in the material, both the phenol/sulphuric acid (Whistler & Wolfrom, 1962) and the anthrone (Scott & Melvin, 1953) colour reactions were employed.

After an initial indication that carbohydrate was a component of the carotenoid glycoprotein, an aliquot containing about 100 mg. was hydrolysed in a sealed tube in N HCl at 100^{o} for 12 hrs. The acid was removed in the usual way and the hydrolysate concentrated to a small volume prior to chromatography. Descending chromatography was carried out on Whatman No.1 paper

using ethyl acetate: pyridine: water (72:20:23) (Colombo, Corbetta Pirotta, Ruffini & Sartori 1960) and n-butanol/pyridine/water (6:4:3) as the developing solvents. To allow for extended development, chromatograms were serrated at their lower edges, and development allowed to proceed for 20 hours. Standards of glucose, mannose, galactose and ribose were also chromatographed, since Salton et al. (1965b) have reported these monosaccharides in the membranes from both S. lutea and M. lysodeikticus. On their removal from the tanks, the chromatograms were dried thoroughly and sprayed with 0.1 M anisidine phthalate (Pridham, 1956) after which they were heated at 100° for 5 - 10 mins. and viewed in daylight. Hexoses appeared as brown spots whilst pentoses were rose-red.

e) Determination of molecular weight by ultracentrifugation

Since the initial examination of the material in the ultracentrifuge had shown a single, symmetrical peak, characteristic of monodisperse system, the approximate molecular weight was determined from the sedimentation coefficient of the carotenoid-glycoprotein. In order to minimise solute-solvent interactions inherent when a protein is dissolved in pure distilled water, and which tend to produce anomalous rates of sedimentation, ultracentrifugation was performed on the material in the presence of a salt solution. An aliquot of known concentration was made approximately 0.1 M with respect to KCl and exhaustively dialysed against 3 changes of exactly 0.1 M KCl for 24 hours in 0.6 inch diameter dialysis sacking. The initial and final volumes of the dialysate were noted in order to allow for any change in

concentration which might have occurred during dialysis. This solution was then examined at 4 different concentrations (dilution of the material being carried out with 0.1 M KCl), in the ultracentrifuge. Schlieren photographs were taken at 20 min. intervals for up to 3 hrs. after the attainment of top rotor speed (59,780 r.p.m.) and the sedimentation coefficient of the material determined from the expression:-

$$S = \frac{dx/dt}{w^2 x}$$

Where : S = sedimentation coefficient ($secs^{-1}$)

x = distance moved by peak (in cms) in time t secs.

w = angular velocity of rotor in radians/sec.

A graph of concentration against S was plotted, and the straight line obtained extrapolated to zero concentration to obtain an approximate absolute value of S for the material, and hence an estimate of the molecular weight.

f) Determination of molecular weight by osmometry

The osmotic pressures of solutions of the material in distilled water at different concentrations were determined using a Hewlett-Packard high speed membrane osmometer. The B 19 membrane was presoaked in distilled water prior to use. All osmotic pressures were measured at 20°, and each represents the mean of two readings.

In order to minimise the Gibbs-Donnan effect which occurs when macromolecules in solution carry a net charge, and which tend to elevate the observed osmotic pressure from the ideal value, osmometry was performed on solutions of the material over a range

concentrations and in the presence of 0.1 M KCl. The adjustment of the concentration of KCl to exactly 0.1 M was carried out in the same way as for ultracentrifugation, by dialysis as above. The osmotic pressures of these solutions were determined in the same manner as above.

For solutions of the material in the presence and absence of KCl, a graph of reduced osmotic pressure (O.P./conc.) against concentration was plotted in each case. To correct for non-ideality of solution, the graph was extrapolated to zero concentration to give an ideal value for the reduced osmotic pressure in the presence and absence of salt.

The molecular weight of the carotenoid glycoprotein under these two conditions was then calculated from the expression:-

$$\bar{M}_n = \frac{RT}{(O.P./conc.)_{c \rightarrow o}}$$

Where : R = gas constant

T = absolute temperature

$\bar{M}_n$ = number average molecular weight

O.p.= osmotic pressure

g) Attempted release of pigment in an ether extractable form

In the case of the water-soluble carotenoproteins extractable from other sources, particularly those from invertebrates (Cheeseman et al., 1967), the carotenoid moiety is easily released from the protein by treatment with protein denaturants, after which it can readily be partitioned into ether. Each of the following procedures was followed by the addition of an equal

volume of diethyl ether and examination of the ether phase spectrophotometrically for the presence of carotenoid pigment :

(i) an equal volume of acetone was added to an aliquot of the material (Cheeseman, 1958)

(ii) an aliquot of the material was made 4 M with respect to urea and stirred overnight at 25°

(iii) the material was made 4M with respect to urea and 1% (v/v) with respect to urea and 1% (v/v) with respect to thioglycollic acid and stirred overnight at 25°

(iv) aliquots of the material were titrated with either 0.1 N HCl or 0.1 N HCl to give solutions at pHs 4.3, 5.3, 7.0, 8.9 amd 11.8.

(v) the material was boiled for 15 mins.

(vi) aliquots were saponified with 10 volumes of methanolic KOH (10% w/v) for periods of up to 7 days at 25°.

(vii) the solution was made : 0.1 M with respect to phosphate buffer, pH 7.6; 1% (w/v) with respect to papain; 5mM with respect to ethylene-diamine tetra-acetic acid (EDTA) to chelate any interfering metal ions present; and 1.0 mM with respect to cysteine to maintain the sulphydryl groups on the enzyme in a reduced form. Digestion was carried out in the dark, under an atmosphere of N_2, at 40° and 2 recharges of papain + EDTA + cysteine made at 5 hour intervals, after which the digestion was allowed to proceed overnight. The etherial extract from the reaction mixture was also tested for the presence of carbo-

hydrate by the method of Whistler et al., (1962), and amino acids by amino acid analysis.

D. CHARACTERISATION OF THE POLAR CAROTENOID FRACTION FROM SARCINA FLAVA

1. Isolation of the polar carotenoid fraction

The isolation of the total pigment, its purification, fractionation by thin layer chromatography and the preparative TLC technique used to isolate the polar carotenoid were all carried out as per Thirkell et al., (1967). All solvents used in these and subsequent procedures were dried and redistilled before use.

2. Further resolution of the polar fraction

The procedure used by Thirkell et al., (1967)for separatin the polar carotenoid fraction into its 4 subfractions (chromatography on kieselguhr-filled circular chromatography paper with 40% acetone in light petroleum, b.p. 60°-80° as solvent) was not adopted in this work. Instead it was decided to develop a preparative T.L.C. method in view of the large quantity of material needed for the chemical analyses. Good separation into the 4 subfractions was obtained using 0.5 mm. layers of silica gel G (Merck) on 20 cm glass plates which were developed using chloroform/methanol, 90:10 as solvent. For the preparative work, the polar fraction was strip-loaded onto the plates, resolved and the bands carefully scraped off the plate, after which the subfractions were eluted from the silica with methanol. The subfractions were respectively designated (i) to (iv) in order of decreasing polarity (increasing R_f) and each was rechromatographed in the same solvent system to

ensure its homogeneity.

Since subfractions (i) and (iii) were present in approximately equal amounts, and together constituted about 80% of the total polar fraction, all the subsequent work was performed on these subfractions only.

3. Analysis of subfractions (i) and (iii).

a) Acetylation

This procedure, first developed by Kuhn and Sorensen (1938) and later modified by Jensen (1962), is used to detect primary and/or secondary hydroxyl functions.

A sample of each of the chromatographically homogeneous subfractions, (i) and (iii), was taken to dryness in vacuo and lyophilised for 24 hours to ensure complete removal of all traces of water. Each was redissolved in 1 ml. pyridine in a stoppered flask and 0.2 ml. acetic anhydride added. The acetylation reaction was allowed to proceed at 25°, in the dark and under an atmosphere of N_2. Small aliquots were withdrawn from the reaction mixture with a micropipette at 30 min. intervals for the first 5 hrs. and thereafter at regular intervals until 36 hrs., after which the reaction was seen to have gone to completion.

As soon as it had been removed from the reaction vessel, each aliquot was immediately chromatographed on 0.25 mm. layers of silica gel G (Merck) with 20% acetone in light petroleum (h.p. 60-80) as solvent. Spots were identified visually in daylight, under ultraviolet illumination and after spraying with a saturated solution of $SbCl_3$ in chloroform (Morton, 1942). In this way, the original compound, the formation of partially esterified inter-

mediates and the final production of the fully acetylated derivative could be monitored. Absorption spectra for both the starting material and the final product were recorded, to ensure that no degradation of the carotenoid had occurred.

b) Trimethylsilylation

This is carried out using the final ester from a) and is a test for a tertiary hydroxyl group. The method used was essentially that of McCormick and Jensen (1966). The final ester from a) was redried as before and redissolved in 0.5 ml. pyridine. 0.2 ml. hexamethyldisilazane + 0.1 ml. trimethylchlorosilane were added and the reaction allowed to proceed under the same conditions as for the acetylation for 1 hr.

After this time, the products were extracted into chloroform, taken to dryness in vacuo, redissolved in a small volume of methanol and examined by T.L.C. as in a).

c) Methylation (Metcalfe and Schmitz, 1961)

This reaction is used as a test for the presence of a carboxyl function. A completely dry sample of material was redissolved in 2 mls. methanol and 0.5 ml. BF_3/methanol complex (BDH Ltd.) was added. The reaction mixture was refluxed for 3 mins. and the reaction stopped by the addition of 2 volumes of distilled water. The products were extracted into ether and examined chromatographically as in a).

d) Partition ratio (Petracek and Zechmeister, 1956)

95% methanol (in water) was equilibrated with hexane in a separating funnel. Each subfraction was dissolved in 5 mls. equilibrated methanol and an equal volume of equilibrated hexane

added. After shaking, the two phases were allowed to separate and the concentration of pigment in each phase estimated by absorbance at 437 nm.

e) Absorption spectra

The absorption spectrum of methanolic solutions of each subfraction and its acetate (from a)) was determined using a Pye Unicam SP 800 spectrophotometer in the ultraviolet and visible regions. The I.R. absorption spectra were also recorded using a Pye Unicam SP 200G instrument, for solutions of the subfractions is CCl_4 and CS_2 (spectral quality reagents).

f) Identification of the carbohydrate moiety

Initial tests using the phenol/H_2SO_4 colour reaction established the presence of carbohydrate in both pigment subfractions and so an attempt was made to identify the monosaccharide(s) released by acid hydrolysis.

The subfractions were each hydrolysed in N HCl for 18 hrs. at 120° after which the hydrolysates were made acid-free in the usual way on the rotary evaporator and reduced to a small volume prior to paper chromatography.

Unidimensional descending chromatography was employed using Whatman No. 1 chromatography paper which was serrated at its lower edge to allow for extended development. Development was allowed to proceed for 22 hours and the following solvent systems were used:-

1. n-butanol/pyridine/water, 6:4:3 (v/v/v)
2. n-butanol/acetic acid/water, 6:1:2 (v/v/v)
3. n-butanol/acetone/water, 4:5:1 (v/v/v).

In view of the observation of Salton *et al.*, (1965b) that membrane hydrolysates from two species closely related to *S. flava*, namely *S. lutea* and *M. lysodeikticus*, contained the monosaccharides glucose, galactose, mannose and ribose, these sugars were employed as standards during the chromatography. These four standards were thus chromatographed separately, and as a mixture and each standard was chromatographed as a mixture with each hydrolysate.

Since earlier in this work it had been shown that some of the carotenoid pigment in the membrane of *S. flava* is associated with a peptide component, in all instances duplicate chromatograms were developed and one sprayed with the anisidine phthalate reagent of Pridham (1956), the other being sprayed with 2% ninhydrin in acetone. In both cases the spray reagents were only applied after thorough drying of the chromatograms. The anisidine phthalate stained chromatograms were heated in an oven at 100° for 5-10 mins. and viewed in daylight and under U.V. illumination. The ninhydrin stained chromatograms were not heated, the spots being allowed to develop slowly for up to 12 hours at 25°.

g) Separation of carbohydrate and peptide components.

Since spraying of the above chromatograms with ninhydrin indicated several components of low R_f value which were almost certainly peptides, as well as 4 components which also stained for carbohydrate, an attempt was made to separate any possibly overlapping spots using 2-dimensional paper chromatography.

A sample of each hydrolysate was applied to Whatman No.1 chromatography paper and duplicate chromatograms were developed using n-butanol/acetic acid/water, 6/2/2 (v/v) in the first dimension. The chromatograms were then dried at 60° for 2 hours before development in the second dimension using phenol/water (400 gms./100 mls). After washing with ether to remove residual phenol, followed by drying, one chromatogram was sprayed with anisidine phthalate, the other with ninhydrin.

h) Quantitative estimation of carbohydrate

As before, the determination of carbohydrate was carried out using the phenol/H_2SO_4 method (Whistler et al., 1962) using solutions of glucose (25-100 µg) as standards. The value for the carbohydrate content of subfraction (iii) was also checked using the anthrone method of Scott et al. (1953).

i) Resolution of peptides

As ninhydrin-positive materials suspected to be peptides were detected on paper chromatography, an attempt to resolve these materials further was made, using a 2-dimensional T.L.C. technique (Randerath, 1964). Aliquots of both N HCl hydrolysates were applied to 0.25 mm. layers of silica gel G (Merck) on 20 x 20 cm. glass plates. The plates were developed in the first dimension using n-butanol/acetic acid/water, 6:2:2 (v/v) as solvent after which they were thoroughly dried before development in the second dimension with n-propanol/water, 64:36 as solvent. After further drying, each plate was sprayed with 2% ninhydrin in acetone and the spots allowed to develop slowly at 25° for up to 12 hrs.

j) Amino acid analysis

10 mg. samples were hydrolysed in 6 N HCl in a sealed tube at 120° for 18 hrs. The hydrolysates were freed from humin and acid in the usual way, redissolved in 1.0 ml. of nor-leucine solution (internal standard) and quantitatively applied to the top of the ion-exchange column of the Technicon amino acid analyser.

k) Estimation of carotenoid

The carotenoid content of the subfractions was determined spectrophotometrically using the theoretical extinction coefficient of Strang (1968), based on the similarity of the chromophore of these pigment subfractions to that of ε-carotene and neurosporene.

E. PREPARATION AND PROPERTIES OF A WATER SOLUBLE PIGMENT FRACTION FROM S. MORRHUAE

1. Isolation

The findings of Nandy et al. (1967) concerning the absorption maxima, chromatographic behaviour and lability of the free carotenoid pigments of S. morrhuae have been confirmed in this laboratory. Thus the major, and probably the only, carotenoid produced by this organism is bacterioruberin.

It was found, however, that even after repeated extraction with methanol, following both prolonged ultrasonication in methanol and saphonification with 10% (w/v) KOH in methanol, the bacterial residue was still well pigmented, although no further pigment could be extracted into methanol, (c.f. Baxter, 1960).

The residue was then stirred at 40° with distilled water for 24 hrs. and a red-orange solution recovered after centrifugation at 2,000 g. for 30 mins. The pellet after centrifugation was

almost colourless indicating that most of the bound pigment had been converted into a water-soluble form.

2. Purification

The material was concentrated prior to desalting on Sephadex by evaporation of water from the outside of a dialysis sack containing the material, using a stream of cold air from an industrial blower.

The concentrated solution was then desalted on a column (45 x 1.5 cm.) of G75 Sephadex. 2 ml. portions of the solution were applied to the top of the column which was eluted with distilled water, at a flow rate of 25-30 mls./hr. 3 ml. fractions were collected and the coloured fractions pooled prior to further desalting and concentration.

The desalted material from the column was placed in an Amicon high pressure ultrafiltration cell and filtered through a UM-2 membrane (molecular weight cut-off, 500), until the volume was reduced to about 10 mls. when the solution was diluted with distilled water and filtration continued. This process was repeated until the filtrate was free of salt as indicated by a negative silver nitrate test for chloride (the major intracellular anion in S. morrhuae being chloride (Christian and Waltho, 1962).

3. Homogeneity of the material

a) Ultracentrifugation

A sample of the desalted, concentrated material was examined in the analytical ultracentrifuge under the same condition as detailed in section C.2.d). The sedimentation pattern was observed and photographed through a Schlieren optical system.

b) Chromatography on Sephadex

1 ml. aliquots were applied to 45 x 1.5 cm. columns of Sephadex G25, G50, G75 and G100 respectively and elution was carried out with 0.1 % NaCl in each case at flow rates in the range 15-25 mls./hr. The void volume of each column was determined using dextran blue, and 3 ml. fractions were monitored for colour and absorption at 276 nm.

c) Agarose column chromatography

A column packed with Biogel A15 (4% agarose) which had been previously calibrated for Stoke's radius with solutions of standard proteins (e.g. horse raddish peroxidase, thyroglobulin, yeast alcohol dehydrogenase and glutamic dehydrogenase) was used. The Stoke's radii of these proteins had been plotted against K_{ave}, to give a calibration graph where:

$$K_{ave} = \frac{(V_x - V_e)}{(V_t - V_e)}$$

K_{ave} = Partition coefficient between the liquid phase and the gel particles.

Where:- V_x = Volume eluted from start to top of sample peak

V_e = Exclusion volume of column

V_t = Total volume of column.

1 ml. of the material was applied to the column which was eluted with 1.0 M potassium acetate. 3.0 ml. fractions were collected and monitored for colour and by absorbance at 276 nm. A graph of effluent volume against absorbance at 276 nm. was plotted which enabled the determination of K_{ave} for the material, and the

estimation of its Stoke's radius from the standard graph.

d) Disc-gel electrophoresis

This was performed on a sample of the purified material under identical conditions to those detailed in section C.2.b).

4. Characterisation of the purified material

a) Estimation of the approximate molecular weight

The approximate molecular weight of the material was assessed from its behaviour on:-

(i) dialysis in visking tubing (approximate molecular weight cut-off 9,000-10,000)

(ii) chromatography on Sephadex G25, G50, G75 and G100 as above

(iii) chromatography on the Biogel A15 column as above

b) Absorption Spectrum

This was recorded for the wavelength range 210-600nm. using the Pye Unicam SP800 instrument.

c) Dry weight and ash determinations

These were determined in the usual manner on duplicate 4.0 ml. aliquots.

d) Protein estimation

Hydrolysates of the material were prepared using 6 N HCl as detailed in previous sections, humin and excess acid were removed in the usual way and protein estimated by two methods:-

(i) Folin-Lowry (Lowry et al., 1951), using bovine serum albumin (0-500 µg) as standard.

(ii) The quantitative ninhydrin method of Moore and Stein

(1954) using solutions of nor-leucine (0-50 µg) as standards.

e) Amino acid analysis

An aliquot of the hydrolysate containing 2.0 mg. protein was taken, made acid free and subjected to analysis on the Technicon analyser as before, using nor-leucine as an internal standard.

f) Carbohydrate estimation

This was performed by the phenol/H_2SO_4 method (Whistler et al., 1962) as before, using solutions of glucose (0-100 µg.) as standards.

g) Carbohydrate identification

A sample of material was hydrolysed in a sealed tube with N HCl at 100 for 12 hours, followed by preparation of the hydrolysate for paper chromatography as before. Descending chromatography was carried out on Whatman No. 1 paper using the following solvent systems:-

Either (i) n-butanol/pyridine/water, 6:4:3 (v/v)

or (ii) ethyl acetate/pyridine/water, 70:20:23 (v/v)

(Colombo et al., 1960).

In both cases the chromatograms were developed for 22 hours, the lower edge of the paper having been serrated as usual to allow for this extended period. Glucose, galactose, mannose and ribose were employed as standards and the hydrolysate was chromatographed alone and as a mixture with each of the standards in turn. As before, duplicate chromatograms were run and after

drying were stained with either the anisidine phthalate reagent of Pridham (1956) or 2% ninhydrin in acetone.

h) Attempted release of free pigment

(i) Acid hydrolysis

5 ml. aliquots of material were made 0.02 N, 0.05 N, 0.1 N and 0.5 N respectively, with respect to HCl and refluxed gently for 15 mins. On cooling, the solutions were extracted with an equal volume of ether and the ether phase examined spectrophotometrically for the presence of pigment.

(ii) Enzymic hydrolysis with papain

A 10 ml. aliquot of the concentrated material was subjected to papain digestion under the same conditions as were used in section C.3.g) (vii) after which the reaction mixture was extracted with an equal volume of ether and the ether phase examined for carotenoid as in (i).

(iii) Enzymic hydrolysis with almond emulsin (crude β-glucosidase)

A 10 ml. aliquot was made: 0.05 N with respect to tris buffer, pH 7.6; 1% with respect to almond emulsin, and the digest was stirred at 30° for 24 hours. The mixture was extracted with ether and the ether phase examined for pigment as in (i).

F. CHEMICAL ANALYSIS OF MEMBRANES PREPARED FROM S. FLAVA CELLS

In all cases, except where otherwise stated, analyses were performed on membranes prepared by the method of Salton *et al.* (1965) from:-

(i) cells harvested after 24 and 57 hours from the same 400 l. culture (at Imperial College)

and (ii) cells harvested after 91 hours from a 12 l. culture (in this laboratory).

Cells harvested at 24 hours were in the late exponential phase of growth whereas the 57 and 91 hour cells were well advanced into the stationary phase.

Thus, although temperature and aeration conditions as well as composition of the growth medium were identical (see section A.2), minor variations such as the speed of agitation and the fact that the 91 hour cells were illuminated during growth and not deep-frozen prior to lyophilisation, may mean that the results obtained for the two earlier ages of membrane may not be strictly comparable with those obtained for the older sample.

1. Dry weight and ash determination

These were determined in the usual way in triplicate for each age of membrane so that the quantitative analyses could be expressed as percentages on a moisture and ash free basis.

2. Carbohydrate analysis

a) Quantitative.

100 mg. samples of membrane were hydrolysed with N HCl in a sealed tube for 12 hours* at 100°. The hydrolysates were centrifuged to remove any residue, which was washed and the washings added to the supernatant. This was then made acid free in the usual way, redissolved in 50 mls. distilled water, and aliquots were withdrawn for estimation of carbohydrate by either:-

(i) the phenol/H_2SO_4 method (Whistler et al. 1962) with solutions of glucose (0-100 µg.) as standards,

or (ii) the anthrone method (Scott et al., 1953) with solution of glucose (0-200 µg.) as standards.

N.B. *12 hours was the hydrolysis time which gave maximal liberation of soluble carbohydrate as indicated by a pilot experiment using the phenol/H_2SO_4 method to assay the released carbohydrate.

The phenol/H_2SO_4 method would seem to be the more reliable for this type of material where several types of compound other than carbohydrate are present. It is pointed out by Whistler et al. (1962) that the anthrone method is interfered with by many compounds e.g. various chromatographic solvents, carbonyl compounds, phenol, toluene, HCl, proteins and tryptophan, so that it is more suitable for determination of samples containing carbohydrate alone. Furthermore, the phenol/H_2SO_4 method is applicable to a wider range of carbohydrates, has a lower % error and the red-brown colour produced is stable for much longer than the green colour characteristic of the anthrone reaction.

b) Qualitative

(i) Paper chromatography

Aliquots of the hydrolysates from a) were reduced to a small volume (0.5 ml.) and applied to Whatman No. 1 chromatography paper, along with the following standards : glucose, galactose, mannose, ribose, arabinose, glucosamine, hydrochloride, galactosamine hydrochloride, mannosamine hydrochloride and N-acetyl glucosamine.

The descending solvent system employed was n-butanol/pyridine/water, 6:4:3 (v/v) and as in previous sections extended

development (22 hrs.) was allowed for by serration of the lower edge of the paper. After drying the chromatograms were stained with anisidine phthalate as before. The $R_{glucose}$ value for each standard and hydrolysis component was determined for this system and the colour of each spot was also noted.

(ii) Gas-liquid chromatography

Aliquots of the acid free N HCl hydrolysates from a), containing about 500 ug. total carbohydrate, were taken and to each was added 0.1 ml. of a standard (1 mh./ml.) solution of ∝-methyl-D-glucoside to act as an internal standard. Each was then taken to dryness in vacuo and redissolved in 1 ml. dry, redistilled pyridine.

Standard solutions in distilled water (1 mg./ml.) of the following sugars were prepared : glucose, galactose, mannose, ribose, arabinose, fucose, rhamnose, N-acetyl glucosamine and glucuronic acid. 0.1 ml. aliquots of each were withdrawn and to each aliquot was also added 0.1 ml. of the ∝-methyl-D-glucoside solution. These standard solutions were then taken to dryness and redissolved in 1 ml. pyridine as above.

Trimethylsilylation of all samples was accomplished by the method of Sweeley, Bentley, Makita and Wells (1963) using hexamethyldisilazane (0.2 mls.) and trimethylchlorosilane (0.02 ml.). The trimethylsilylated hydrolysates were then taken to dryness in vacuo and redissolved in 0.5 ml. dichloromethane since this solvent induces a smaller detector response, and hence gives a smaller solvent peak, than pyridine on GLC.

Chromatography was performed on a Pye Model 104 Gas

Chromatograph using twin columns and under the following conditions

Columns : glass, 5 foot, containing 10% E 30 (methyl silicone gum) on 100-120 mesh celite.

Temperature programme : 170° (8 mins.) X 4°/min. to 190° (5 mins.).

Detector oven temperature : 210°.

Injection point heater : 220°.

Attenuation : 10×10^2.

Carrier gas (N_2) flow rate : 45 ml./min.

Air flow rate (to detector) : 600 ml./min.

H_2 flow rate (to detector) : 40 ml./min.

Chart speed : 10 mm./min.

Sample size : 1 µl.

Retention distances were determined for the standards and for the hydrolysate components and the $^{R}\alpha$-methyl glucoside calculated in each case to allow for any slight non-reproducibilit which might occur between separations.

3. Lipid analysis

a) Extraction, purification and estimation of yield of lipid fractions

A variety of trial extraction schemes were attempted, but the one which gave the greatest yield of total lipid, and which was hence adopted for this work is a modification of that used by Huston and Albro (1964) for the extraction of lipids from S. lutes This scheme is shown in Fig. M. 2.

N.B. 1. It was found that the yield of lipid was augmented significantly by moistening the membranes prior to

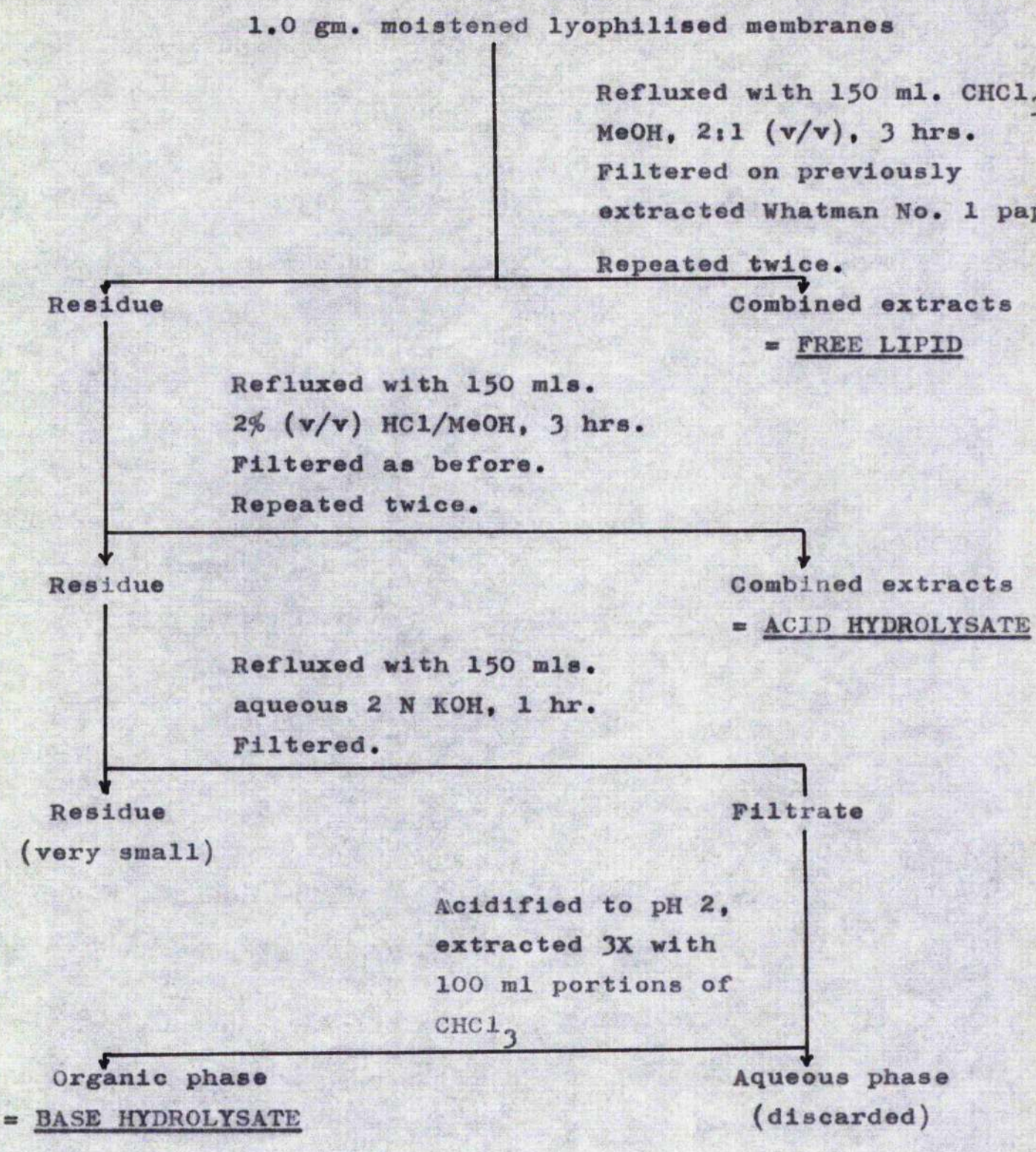


Fig. M. 2 - Extraction Scheme for S. flava Membrane Lipids

extraction. 10 mls. distilled water were added to each 1 gm. portion of lyophilised membranes and these were allowed to swell for 1 hour before the extraction procedure was begun.

2. Both the basic and acidic hydrolytic steps in the scheme will produce some degradation of the complex lipids, yieldin water-soluble hydrolysis products e.g. serine, ethanolamine and hence the figures for the proportion of the total lipid extracted by these steps will be depressed. These fractions represent (protein?) bound lipid which cannot be extracted using organic solvents alone.

Non-lipid contaminants were removed from the lipid samples by the method of Folch, Lees and Sloane-Stanley (1957), as follows:

Each fraction was taken to dryness in vacuo and redissolved in 500 mls. chloroform/methanol, 2:1 (v/v) and shaken in a separating funnel with 100 mls. 0.05 N NaCl. The washed lipids in the hypophase were dried in vacuo and lyophilised to constant weight, the weight of each fraction being accurately determined.

b) Fatty acid composition of membrane lipid fractions

(i) Isolation of fatty acids and preparation of methyl esters for GLC

Each fraction was redissolved in 100 mls. 10% (w/v) KOH/MeOH and saponified overnight at 25°. Each saponification was diluted with 2 volumes distilled water and extracted 3 times with ether. The combined ether extracts, containing the non-saponifiable lipids were dried over anhydrous Na_2SO_4 and stored at 0° until further investigation.

The aqueous phase from the ether extraction was acidified

to pH 2 by careful addition of conc. HCl and extracted 3 times with ether. The combined ether extracts, containing the fatty acids were dried over anhydrous Na_2SO_4 and the ether removed in vacuo.

Esterification was carried out by redissolving the fatty acids in 25 ml. BF_3/MeOH complex (BDH Ltd.) and refluxing for 15 mins. The reaction was stopped by pouring the reaction mixture into two volumes distilled water and the fatty acid methyl esters extracted into ether (3 extractions) after the addition of a small amount of solid NaCl to the aqueous phase.

The combined ethereal extracts were washed firstly with 1% $NaHCO_3$ (to remove traces of HF formed by the hydrolysis of the BF_3), and secondly with distilled water. The solution of methyl esters was then dried over anhydrous Na_2SO_4 and concentrated to a small volume (not more than 0.25 ml.) prior to GLC analysis.

A mixture of standard fatty acid methyl esters was also prepared by dissolving 10 mg. of each of the following fatty acids (BDH Ltd.) in 50 mls. BF_3/MeOH complex and proceeding as per the above method:-

n-octoic (8:0); n-decoic (10:0); lauric (12:0); myristic (14:0); palmitic (16:0) and stearic (18:0).

(ii) Catalytic reduction of fatty acid methyl esters

This was performed on a small aliquot of the methyl esters from each fraction, dissolved in absolute ethanol. Reduction was accomplished by shaking the solution in an atmosphere of H_2, in the presence of a palladium/charcoal catalyst for 30 mins at 25°. The reduced methyl esters were then reextracted into ether in the usual way and concentrated as before prior to GLC analysis.

(iii) Gas-liquid chromatography of fatty acid methyl esters

The instrument used was a Pye Model 104 Gas Chromatograph, fitted with a single column and analysis was carried out under the following conditions:-

Column: 5 foot, stainless steel, containing either 10% diethyleneglycol succinate (DEGS) or 5% Apiezon L (APL) both on 100-120 mesh celite.

Temperature: 100° (DEGS) or 215° (PL), both columns being operated isothermally.

Detector oven temperature: 210° (DEGS) or 235° (APL).

Injection point heater: 220° (DEGS) or 245° (APL).

Attenuation: 50×10^2.

Carrier gas (N_2) flow rate: 45 ml./min.

Air flow rate (to detector): 600 ml./min.

H_2 flow rate (to detector): 40 ml/min.

Chart speed: 25 mm./min.

Sample size: 1µl.

(iv) Identification and quantitation of fatty acid methyl esters

The unknown methyl esters were tentatively characterised from their carbon numbers which were determined using plots of carbon number versus logarithm of retention distance for the standard methyl esters. Additional identification was obtained by comparing retention times with those previously reported by Burchfield and Storrs (1962), for both the polar (DEGS) and the non-polar (APL) columns.

Any unsaturated components which were present were

identified by comparison of their retention times before and after reduction.

The proportions of fatty acid methyl esters present in any one fraction were estimated by relating individual peak areas to the total area. Individual peak areas of fully resolved component were determined from the product of the peak height and the retention distance, since this method is more convenient and just as accurate (for structurally related compounds such as fatty acid methyl esters), as any of the other commonly used techniques for determining peak areas e.g. planimetry, triangulation and peak weight (Gough and Walker, 1969). Incompletely resolved peaks were resolved, and their areas determined using an electronic curve analyser (DuPont Ltd.).

c) Examination of the non-saponifiable fraction for steroid

With the exception of the Mycoplasma and certain of the Streptococcal L-forms, the absence of sterols is characteristic of bacterial lipids in general. However, Strang (1968), during his investigation into the carotenoid pigments of S. flava, reported the presence of a component in the unsaponifiable lipid fraction which migrated with the same R_f value as cholesterol on TLC, and gave the pink colour when stained with $SbCl_3/CHCl_3$ which is characteristic of steroids.

Thus the following work was done on the unsaponifiable lipid material, obtained during the isolation of the fatty acids, from the three lipid fractions as above.

(i) Thin-layer chromatography

This was carried out on the free and acid hydrolysed

lipid fractions from 24, 57 and 91 hour membranes. The dry unsaponifiable lipid solutions were reduced to a small volume and aliquots applied to 0.25 mm. layers of Silica gel G (Merck) on 20 x 20 cm. glass plates. The plates were developed with benzene/ethyl acetate, 5:1 (v/v) (Avigan, Goodman and Steinberg, 1963) for 1 hour. Standards of cholesterol and cholesterol stearate were also chromatographed on the same plate. After drying, duplicate chromatograms were sprayed with either:-

I. 0.05% (w/v) rhodamine 6G in ethanol, followed by viewing under U.V. illumination.

II. a saturated solution of $SbCl_3$ in $CHCl_3$, followed by examination in daylight.

(ii) Gas-liquid chromatography

As a check on the results obtained by TLC the unsaponifiable lipids from the 3 lipid fractions (free, acid hydrolysate & base hydrolysate), for each of the three ages of membrane, were subjected to GLC analysis under optimum conditions for the separation of sterols.

Standard steroids were also chromatographed:-

(1) Pregnanediol - 3β, 22β

(2) Cholesterol

(3) Progesterone

(4) Testosterone

(5) Dehydroisoandrosterone.

Chromatography was carried out on a Pye Model 104 Gas Chromatograph with twin columns, under the following conditions:-

Columns: 5 foot, glass, containing 10% E30 on 100-120 mesh celite.

Temperature: 250° isothermal.

Detector oven temperature: 270°.

Injection point heater: 280°.

Attenuation: 10×10^2.

Carrier gas (N_2) flow rate: 45 ml./min.

Air flow rate (to detector) : 600 ml./min.

H_2 flow rate (to detector): 40 ml./min.

Chart speed: 10 mm./min.

Sample size: 1µl.

4. Amino acid analysis

100 mg. samples of each age of membrane were hydrolysed for amino acid analysis as in previous sections. After removal of humin and acid in the usual way, aliquots of each hydrolysate containing approximately 1 mg. protein were analysed on the Technicon autoanalyser, using norleucine as an internal standard.

5. Determination of total phosphorus content

Total phosphorus was determined by the method of Fiske and Subbarow (1925) which was modified for use with a Technicon autoanalyser system by Holman (1969). Variations in the intensity of the colour often encountered with this method were overcome in this system by:-

(i) heating reagents and samples at 100° for 5 mins.

(ii) ensuring that the time between mixing of reagents and sample and the spectrophotometric reading was

identical for each determination. The method was extremely sensitive (0-10 µg. P) and highly reproducibl

Reagents: 1. Ammonium molybdate reagent - 25 gms. ammoniu molybdate dissolved in 300 mls. 10N H_2SO_4, made up to 1 l. with distilled water.

2. Aminonaphtholsulphonic acid (ANSA) reagent - 0.125 gms. 1,2,4-aminonaphtholsulphonic acid added to 195 mls. 5% $NaHSO_3$ solution and 1 ml. portions 20% Na_2SO_3 solution added until all the aminonaphtholsulphonic acid was in solution. (Addition of excess sodium sulphite at this point must be avoided since the acidity resulting from this renders the reagent less stable). Th ANSA reagent was found to be stable at 4° for up to 4 weeks, and was always filtered immediately before use.

Method: Triplicate 30 mg. samples of membrane were weighed into Kjeldhal digestion tubes and each digested with 6.0 mls. 10N H_2SO_4 on an electrically heated rack for 12 hours. Any colour which remained in the digests after this time was removed by the addition of a few drops 10 vol. H_2O_2, followed by further heating. Each hydrolysate was made up to 50 mls. with distilled water and duplicate 2.0 ml aliquots withdrawn from each for P determination. Duplicate standard solutions of Na_2HPO_4, containing 2.5, 5.0, 7.5 and 10.0 µg. P/ml. were also analysed. The

samples and standards were placed in an automatic sampler which operated at a rate of 60 samples/hour. Each test solution was mixed with the reagents by means of a multi-channel peristaltic pump at the following flow rates:-

Sample	0.8 ml./min.
Ammonium molybdate	0.42 ml./min.
ANSA reagent	0.16 ml./min.
Water	1.2 ml./min.
Air	0.60 ml./min.

After mixing the reaction mixture was passed through a heating coil, a delay coil and finally through the spectrophotometer where the colour developed was read at 662 nm.

6. RNA estimation

This was performed using the orcinol method of Schneider (1957). A step for the removal of DNA was not included since the preparation procedure employed in this work involved the treatment of the membranes with DNAase.

Orcinol reagent: 1.0 gm. orcinol dissolved in 100 mls. conc. HCl containing 0.5 gm. $FeCl_3$.

Method: 500 mg. portions of membrane were defatted by prolonged (24 hrs.) extraction with methanol in a soxhlet apparatus after which the defatted residue was dried thoroughly. 100 mg. samples of defatted membrane were suspended in 2.5 mls. cold 10% (w/v) trichloroacetic acid (TCA), centrifuged, the supernatant discarded and the pellet washed again with the same volume of 10% TCA. The pellet was then suspended in 2.6 mls. 5% TCA, heated

at 90° for 15 mins. with stirring and centrifuged. The supernatant was retained and the pellet washed with a further 2.5 mls. 5% TCA followed by centrifugation. The combined supernatants (5.0 mls.) contain the total RNA (and DNA if present) of the sample.

Duplicate 0.2 ml. aliquots of the RNA extract from each membrane sample were diluted to 1.5 mls. and each heated with 1.5 mls. orcinol reagent for 20 mins. at 100°. The green colour which developed was read at 660 nm. as soon as the assay mixtures had cooled to ambient temperature. A calibration curve was prepared at the same time using solutions of ribose (0-100 µg.) as standards.

6. ELECTRON MICROSCOPIC EXAMINATION OF S. FLAVA AND S. MORRHUAE

1. Methods of fixation

Several methods of fixation were employed on the following specimens:-

(i) *S. flava* cells grown in nutrient broth for 48 hours, as detailed in section A.2.a).

(ii) Protoplasts prepared as in section B.3. from *S. flava* cells grown for 60 hours under the conditions indicated in section A.2.a).

(iii) *S. morrhuae* cells grown for 3 weeks on the solid medium and under the conditions detailed in section A.4.

a) Osmium tetroxide method I.

This method was essentially that used by Chapman (1960),

in his ultrastructural study of S. lutea, and was used here to fix S. flava cells grown for 48 hours in nutrient broth as above.

Reagents: Solution 1 = 2.5 mls. acetate buffer, pH 9.0 (9.71 gms. $NaAc.3H_2O$ + 14.71 gms. sodium barbitone made up to 500 mls. in distilled water); 1.85 mls. 8.5% (w/v) NaCl solution; 3 drops 0.11 M $CaCl_2$ and 2.75 mls. distilled water.

Solution B = 2% (w/v) OsO_4 in distilled water.

Fixative solution = 5.7 mls. A + 5.0 mls. B

Procedure: The bacterial pellet (500 mg. wet weight approx.) was suspended in 10 mls. fixative for 4 hours at 25° after which the suspension was centrifuged at 1,500 g. for 30 mins. The pellet of fixed bacteria was washed on the centrifuge with solution A and the resultant pellet was then dehydrated as detailed below.

b) Osmium tetroxide method II (after Kellenberger, Ryter & Sechaud 1958)

This method was adopted as a standard method for the fixation of bacteria after a systematic study, by Ryter & Kellenberger (1958), of the response of bacterial nuclear material to different fixation conditions. As in method I it is necessary to add either Ca^{++} or Mg^{++} since the standard osmium fixation technique for animal tissue (Palade, 1952), of which both method I and II are modifications, does not preserve the nuclear elements of bacteria well.

The method was used here to fix whole cells of S. flava grown in nutrient broth for 48 hours as before, and whole cells of S. morrhuae grown for 3 weeks on the solid medium as previously

described.

Reagents: Solution A = veronal-acetate buffer (2.94 gms. sodium barbitone + 1.94 gms. $NaAc.3H_2O$ + 3.40 gms NaCl made up to 100 mls. with distilled water).

Solution B = Kellenberger Buffer - 5.0 mls. A + 13.0 mls. distilled water + 7.0 mls. 0.1 N HCl + 0.25 mls M $CaCl_2$, pH adjusted to 6.0 with HCl, prepared freshly as required.

Fixative solution : 0.1 gm OsO_4 dissolved in 10 mls. Solution B (pH remains constant).

Washing fluid : 0.5 gm. uranyl acetate in 100 ml B.

Tryptone medium : 1.0 gm. tryptone (Oxoid Ltd.) + 0.5 gm. NaCl in 100 mls. distilled water.

Procedure : N.B. The prefixation step of Kellenberger et al (1958) was omitted in this case.

500 mg. wet weight bacteria were suspended in 5 mls. fixative + 0.5 ml. tryptone medium and the fixation allowed to proceed overnight (16 hrs.) in a stoppered bottle at 25°. The suspension was diluted with 8.0 mls. Solution B and centrifuged. The pellet was resuspended in a small drop of molten agar, which was then allowed to set on a microscope slide. When firm, the agar was cut into cubes (1.0 mm^3 approx.) and the cubes placed in the washing fluid for 2 hours at 25, after which they were dehydrated as below.

Modification for the fixation of S. flava protoplasts

1. All procedures were carried out at 4°

2. The method was modified after Highton (1969) in that KCN was added to Solution B, to a final concentration of 0.04 M

3. In view of the osmotic fragility of the protoplasts, sucrose was included in both solution B and the tryptone medium, to final concentration of 0.75 M.

4. In order to determine the effect of pH on the fixation obtained by this method, 3 different batches of protoplasts were fixed for which:

   a) the pH of solution B was adjusted to 8.0

   b) the fixation was carried out normally at pH 6.0 (with the modifications listed above).

   c) the veronal-acetate buffer component of solution B was replaced by citrate-phosphate buffer pH 4.0, of the same ionic strength.

c) Osmium tetroxide/glutaraldehyde double fixation

It has been shown (Ledbetter & Porter, 1963; Sabatini, Miller & Barrnett, 1964) that the preservation of biological material fixed with glutaraldehyde and osmium tetroxide together is usually an improvement on that obtained with osmium tetroxide alone. Furthermore, some components e.g. membranes, in tissues treated with aldehyde fixatives alone, tend to be extracted during subsequent dehydration procedures unless the primary aldehyde fixation is followed with a secondary osmium fixation (Sabatini, Bensch & Barrnett, 1963).

This method was used to fix whole cells of S. flava and S. morrhuae grown under the conditions indicated at the beginning of this section.

Reagents : Solution A = 13.6 gms. KH_2PO_4 made up to 1 l.

with distilled water (= 0.1 M solution).

Solution B = 17.8 gms. $Na_2HPO_4.2H_2O$ made up to 1 l. with distilled water (= 0.1 M solution).

Solution C = 20 mls. A + 80 mls. B + 7.5 gms. sucrose (= Sorensens double phosphate buffer + 0.22 M sucrose).

Fixative solution = 50 mls solution C + 4.0 mls. 25% (w/v), glutaraldehyde.

Procedure : All operations were carried out at 4°. 500 mgs. wet weight bacteria were suspended in 5.0 mls. fixative for 3 hours. The pellet obtained after centrifugation was washed twice with Solution C and then subjected to fixation by the previous method (osmium tetroxide II), but for 2.5 hours only.

d) Permanganate fixation (modified after Luft, 1956)

Fixation with buffered $KMnO_4$ is often used as an alternative to OsO_4 since, unlike OsO_4 it is not lipophilic. It is however a strong oxidising agent and is usually used at low temperatures. Even under these conditions, considerable destruction of some components does occur e.g. particles with a high RNA content and myofilaments of striated muscle. Nuclear material is not well preserved after fixation by this method.

Whole cells of S. flava and S. morrhuae, grown as before, were fixed by this method.

Reagents : Solution A = veronal-acetate buffer (14.7 gms. sodium barbitone + 9.7 gms. $NaAc.3H_2O$, made up to 500 mls. with distilled water).

Solution B = 1.2% (w/v) $KMnO_4$ in distilled water.

Fixative solution : 1 vol. A + 1 vol. B, pH adjusted to 7.4 by addition of 0.1 N HCl.

Procedure : All operations were carried out at 4°. 500 mgs. wet weight bacteria were suspended in 5 mls. fixative for 2 hrs., centrifuged and the pellet washed twice in Solution A. The pellet was then ready for dehydration as below.

Modification for the fixation of S. flava protoplasts

1. Owing to the powerful oxidising action of $KMnO_4$, sucrose could not be used to raise the osmolarity of the fixative so that KCl was added to a final concentration of 0.375 M.
2. The final pellet was suspended in agar as previously detailed prior to dehydration.
3. Three different batches of protoplasts were fixed by this modified method at pHs 4.0, 6.0 and 8.0 respectively, as before.

2. Dehydration

Dehydration of fixed material was achieved by immersing the specimens in a series of alcohol/water mixtures, of gradually increasing alcohol concentration.

a) Method for bacterial pellet

The pellet was suspended in the following series of alcohol/water mixtures, for the times indicated, in a 50 ml. glass centrifuge tube. The suspension was centrifuged at each stage before the addition of the stronger alcohol solution, the supernatant being discarded each time.

% ethanol	Time in suspension
20	10 mins.
50	10 mins.
70	10 mins.
95	15 mins.
100	30 mins.
100	30 mins.

Finally the pellet was suspended in two changes of 100% epoxypropane, for 30 mins. in each, after which the material was ready for embedding.

b) Method for agar cubes

The method employed for the dehydration of the S. flava protoplasts suspended in agar cubes was essentially the same as that for the bacterial pellet, but since the cubes can be handled by forceps, and the dehydrating solutions can be easily decanted off, the need for centrifugation is eliminated.

3. Embedding

Two epoxy resins were used for embedding the dehydrated specimens:-

a) Araldite - (CIBA Ltd.)

The method used was modified after that of Glauert and Glauert (1958).

Materials : A = Araldite epoxy resin CY212

B = Araldite hardener HY964

C = Araldite accelerator DY064

D = Dibutyl phthalate

Preparation of embedding medium : 10 gms. A + 10 gms B were mixed by inversion overnight, (= A + B). 10 gms C + 10 gms. D were also mixed by inversion for 2 hrs., (= C + D), after which C + D was stored at 4° where it remains stable for some weeks. The final embedding medium was prepared by mixing 19 gms. A + B and 1 gm. C + D by inversion for 6 hrs. after which it was ready for use.

Embedding of fixed, dehydrated bacteria and protoplasts : The dehydrated material was suspended in 50% ABCD/50% epoxypropane (v/v) overnight, followed by 100% ABCD for 1 hr. After resuspension in another batch of ABCD, the specimens were transferred to either BEEM capsules or gelatin capsules and the araldite polymerised at 60° for 48 hrs.

For pellets of bacteria, centrifugation was again necessar between successive additions of embedding medium. The agar cubes were merely removed from one batch of medium and blotted on filter paper before transfer to the next with a pair of fine forceps.

b) Maraglas (Minnesota Mining & Manufacturing Corporation)

The method used was basically that of Freeman and Spurlock (1962).

Materials : A = Maraglas 655 epoxy resin
B = Cardolite NC513 hardener
C = Dibutyl phthalate (plasticiser)
D = Benzyldimethylamine (accelerator)

Preparation of embedding medium : 32.5 mls. A + 15 mls B were measured out into a stoppered glass measuring cylinder and

2 mls. C + 1 ml. D pipetted into the same vessel. The final embedding medium, ABCD, was ready for use after mixture by inversion for 1 hr. This medium was found to be lighter in colour and less viscous than Araldite.

Embedding of protoplasts : The agar cubes containing the fixed, dehydrated specimens were suspended in 50% ABCD/50% epoxypropane (v/v), for 2 hrs., then 100% ABCD overnight, followed by suspension in a further batch of 100% ABCD and transfer to BEEM or gelatin capsules prior to polymerisation at 60° for 48 hrs.

4. Ultramicrotomy

Blocks from the BEEM or gelatin capsules were trimmed with a sharp razor blade, to give the smallest trapezoidal block face possible, under a binocular microscope. The block was then mounted in an LKB Ultrotome I.

45° glass knives were prepared from strips of LKB glass (25 mm.), using an LKB knife maker equipped with a break-damping device. Silver and grey sections were collected on either 2% (v/v) ethanol or 10% (v/v) acetone. After being stretched by exposure to xylol vapour, the sections were collected on uncoated copper grids (100 or 200 mesh. Polaron Equipment Ltd.).

5. Post-staining

Two post-staining methods were employed, and all stains and washing solutions were Millipore filtered through a 0.45 μ pore size membrane prior to use.

a) Single staining with uranyl acetate

Uranyl ions, under certain conditions, preferentially

stain the DNA of the cell (Huxley & Zubay, 1961). This differential effect being more pronounced when the material is subsequently stained with lead hydroxide. In addition, it has been shown that 0.5-1.0% (w/v) aqueous uranyl acetate stains deoxyribonucleoprotein in preference to ribonucleoprotein, in tissues fixed with acrolein (Mannozzi, 1963) or formaldehyde (Mannozzi & Gautier, 1962).

Procedure : Grids were floated, sections downwards, on 2% (w/v) uranyl acetate in 50% aqueous ethanol for 20 mins. The sections were then washed by floating on distilled water, and dried by placing the grids, sections uppermost, on filter paper.

b) Double-staining with uranyl acetate and lead citrate

Staining with lead at high pH is a rapid process which increases contrast generally although it has been shown (Daems & Persijn, 1963) that lead had an increased affinity for RNA-containing structures.

Staining reagents : (i) 2% (w/v) uranyl acetate in 50% aqueous ethanol, as above.

(ii) Reynolds' lead citrate stain (1963) prepared as follows : 1.33 gms. $Pb(NO_3)_2$ + 1.76 gms. $Na_3(C_6H_5O_7).2H_2O$ + 30 mls. distilled water were placed in a 50 ml. volumetric flask, shaken vigorously for 1 min. and allowed to stand for 30 mins. (to ensure complete conversion of the lead nitrate to lead citrate). 8.0 mls. N NaOH were added, the solution was made up to 50 mls with distilled water and filtered. This stain is stable for 6 months.

Procedure : Sections were first stained with uranyl acetate as above. After washing with distilled water, the grids were floated, sections downwards on the lead citrate stain for up to 5 mins*, washed with 0.02 N NaOH and finally with distilled water. After drying on filter paper as above, the sections were ready for examination in the electron microscope.

N.B. *Normally the recommended staining time for sections cut from material embedded in an epoxy resin, using Reynold's lead citrate stain is said to be 15-30 mins. However, after fixation with phosphate-buffered osmium tetroxide (Millonig, 1961) or glutaraldehyde (Sabatini et al., 1963), the intensity of this stain is much increased. Thus the staining time was reduced for sections from material fixed in either of these ways.

6. Electron microscopy and photography

a) Electron microscopy

This was carried out on an A.E.I. EM6B instrument with an accelerating voltage of 60 kV, an objective aperture of 50 μ. and a condenser aperture of 250 μ.

b) Photography

All photographs were taken at an instrumental magnification of 30,000 and enlarged further photographically where deemed necessary. Exposure time was 0.5 sec. for a photometer reading of 9 and the photographic plates used were either Ilford N50 (thin film, half tone) or Ilford EM3.

RESULTS

A. THE EFFECT OF LIGHT ON THE GROWTH AND PIGMENTATION OF S. FLAVA

The growth curves for both light and dark-grown cells, as estimated by turbimetric measurements, are shown in Fig. R.1. Maximum numbers were reached, in both cases, after 26 hours but the total number of dark-grown cells was only 48% that of the light-grown cells at this time. In addition, the dark-grown cells entered the phase of decline far sooner so that after 7 days, the optical density of the culture was practically zero whereas for the light-grown cells, the optical density was still 85% of that at 26 hours.

At 26 hours, the light-grown cells were found to contain 20% more carotenoid pigment/unit bacterial dry weight than the dark-grown cells.

B. HOMOGENEITY AND PROPERTIES OF CRILLET 1 and LUBROL L SOLUBILISED CAROTENOID GLYCOPROTEINS FROM S. FLAVA MEMBRANES

1. Homogeneity

a) Electrophoresis in free buffer film

In no buffer system employed, and at no pH between 3.9 and 8.6 did either material separate into more than one component. An apparent lack of appreciable net charge at any pH was indicated since the materials did not deviate significantly from a vertical elution path.

b) Disc-gel electrophoresis

Both preparations gave a single discrete band with a negligible mobility relative to the standards used (lysozyme and bovine serum albumin), under the conditions of the experiment.

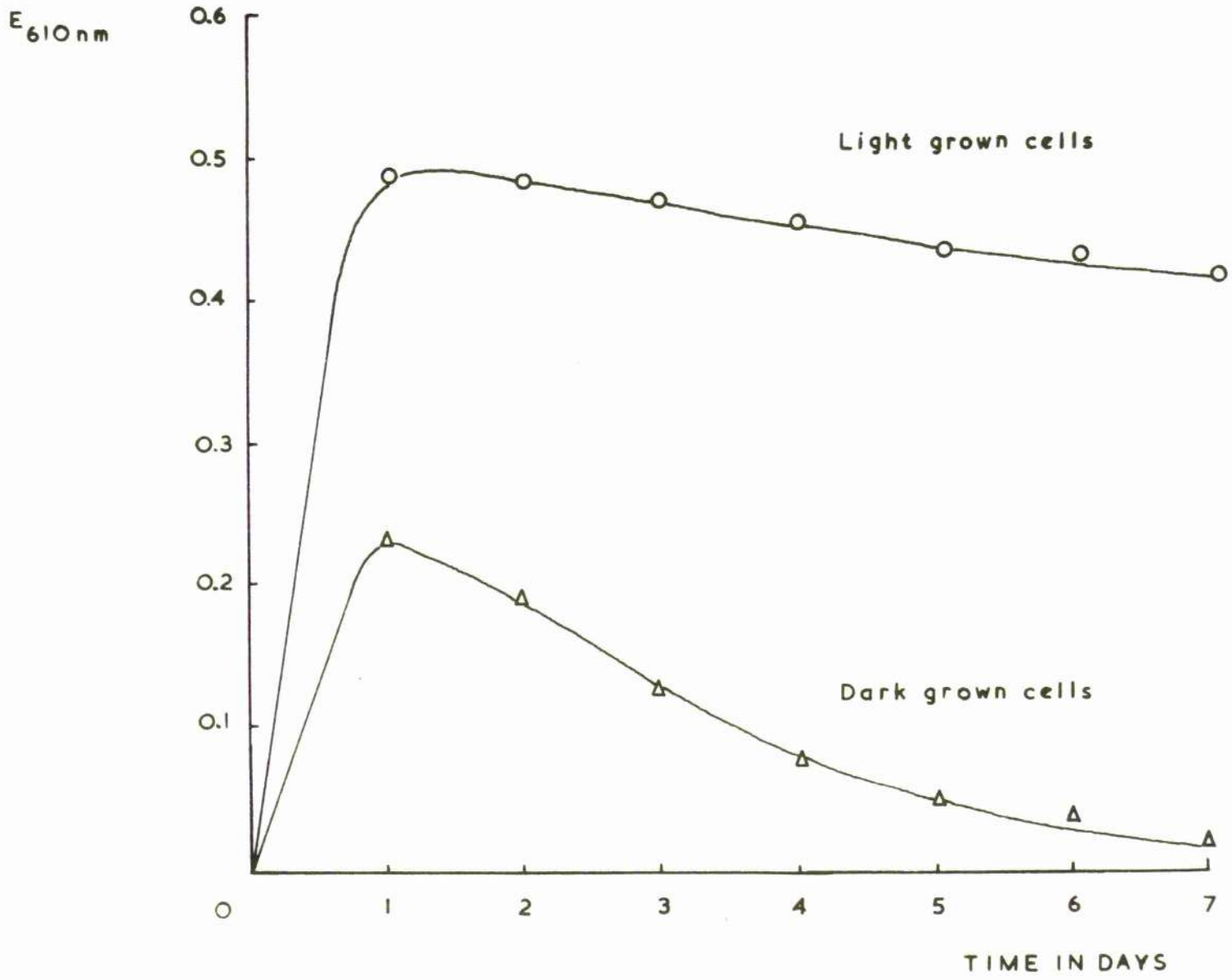


FIG.R.1. Growth curves of light and dark grown S. flava cells

c) Sephadex gel-filtration

Neither material was resolved on either G100 or G200 into more than one component as verified by both absorbance at 444 nm. and by Folin-Lowry estimation.

d) Ultracentrifugation

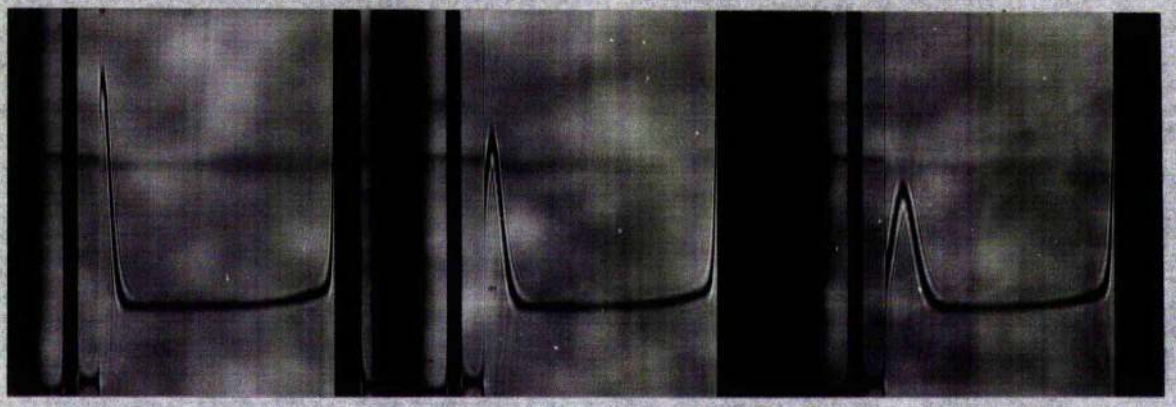
For both types of material, a single, symmetrical peak was observed on the analytical ultracentrifuge, 1 hour after attainment of maximum rotor speed (59,780 r.p.m.). The Schlieren pattern produced by the Lubrol L solubilised material is shown in Fig. R.2. The symmetry of the peak and the total absence at any stage of the run of even small additional peaks or shoulders indicates that the material is certainly a monodisperse system of molecules, if not a single molecular species.

Attempts to examine the sedimentation behaviour of the Lubrol L solubilised material in the presence of 4M urea were unsuccessful due to the extreme density of the solution, which caused the production of an equilibrium pattern on ultracentrifugation.

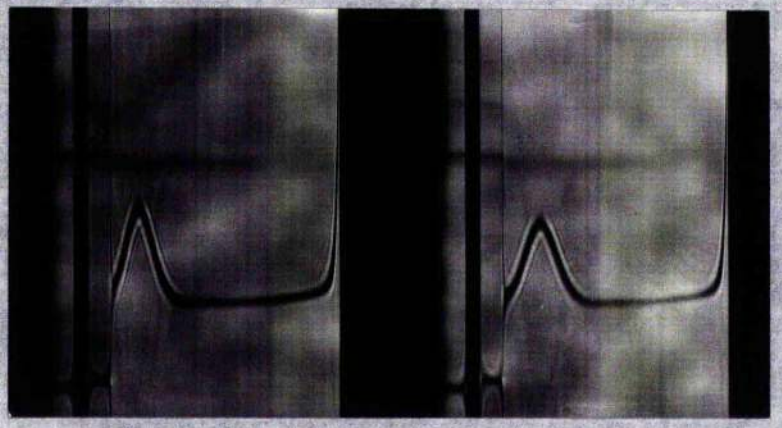
2. Properties of the Lubrol L solubilised carotenoid glycoprotein

a) Absorption spectrum

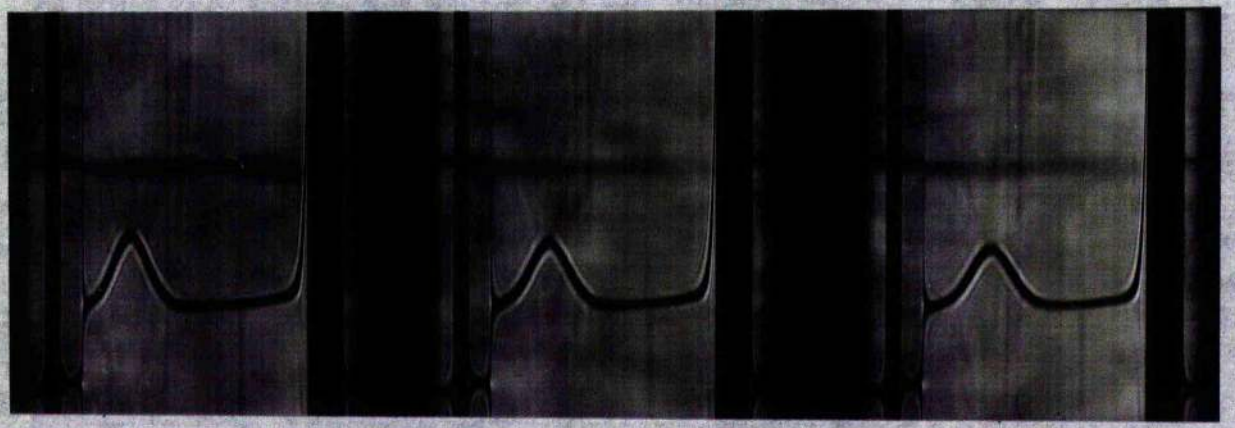
The absorption spectrum is shown in Fig. R.3. Maxima were observed at 226, 276, 282, 297, 416, 444, and 474 nm. The maxima in the visible region are due to carotenoid, and show only slight deviation from those of the free pigments from the same organism in organic solvents, suggesting that the binding of the carotenoid to other molecules does not significantly alter the chromophore of the pigment. This is not the case for caroteno-



1 2 3



4 5



6 7 8

Fig. R.2. - Schlieren pattern produced by Lubrol L solubilised carotenoid glycoprotein.

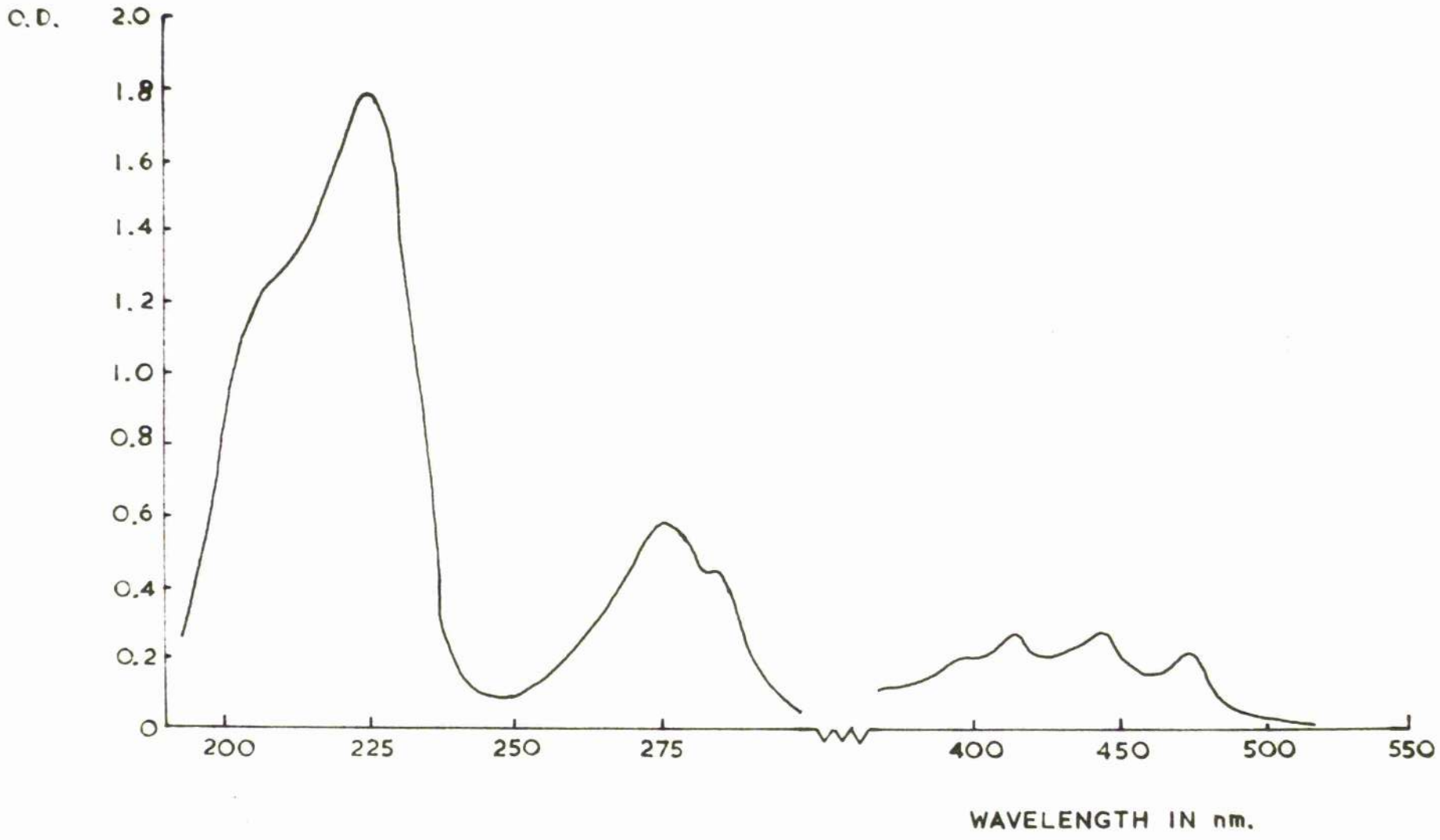


FIG R.3. Absorption spectrum of Lubrol L solubilised material (U.V. dil. X50)

proteins isolated from invertebrates (Cheesman et al., 1967).

No change was observed in either the position of absorption maxima or peak heights over the ranges of temperature and pH employed, and prolonged boiling did not result in any turbidity or precipitation. These observations substantiate the findings of Cheesman et al., (1967) that the association of protein and carotenoid is accompanied by mutual stabilisation of both components.

b) Optical rotation

The material had a negligible optical rotation with reference to distilled water and this remained unchanged throughout the temperature range employed, suggesting that no alteration in the overall conformation of the carotenoid glycoprotein occurred.

c) Amino acid composition

The results of the amino acid analysis are shown in Table R.1. below:-

Amino acid	% Total amino acids
Alanine	9.7
Aspartic acid	8.7
Glutamic acid	8.0
Glycine	9.8
Histidine	5.3
Isoleucine	8.2
Leucine	14.2
Lysine	Trace
Ornithine	Trace
Phenyalanine	6.7
Serine	10.4
Threonine	6.0
Tyrosine	4.0
Valine	9.0

Table R.1. - Amino acid composition of carotenoid glycoprotein from S. flava membranes.

The amino acid pattern shows the characteristic trends common to most membrane proteins viz. a high proportion of the acidic and apolar residues and a low content of the basic and sulphur-containing amino acids. The very large ammonia peak on the analyser trace suggests that the aspartic and glutamic acid residues were present largely as their respective amides.

d) Identification of carbohydrate.

Using either solvent system, only one monosaccharide could be detected using anisidine phthalate. This component possessed the same R_f value as glucose and this was confirmed by co-chromatography in both solvent systems.

The $R_{glucose}$ values of the standards, for the system : ethyl acetate/pyridine/water, 72:20:23 (v/v/v) are shown below in Table R.2.:-

Standard	$R_{glucose}$	Colour of spot
Glucose	1.00	Brown
Mannose	1.25	Brown
Galactose	0.84	Brown
Ribose	2.16	Rose-red

Table R.2: $R_{glucose}$ values of standard sugars in the descending solvent system ethylacetate/pyridine/water, 72:20:25 (v/v/v).

The $R_{glucose}$ values for these and other standards chromatographed using the system: n-butanol/pyridine/water, 6:4:3 (v/v/v) are shown in a later section in Table R.20.

e) Determination of sedimentation coefficient and molecular weight by ultracentrifugation.

The Schlieren pattern produced by the material is that shown in Fig. R.2., where the interval between photographs is 20 mins.

The sedimentation data for 4 different concentrations of the material in the presence of 0.1 M KCl are presented in the plot-shown in Fig. R.2.a. From this graph, S_{20} (extrapolated) = 1.49×10^{-13} secs. and by comparison with S_{20} values for proteins of known molecular weight (see Table R.3. below), the molecular weight of the carotenoid glycoprotein is probably about 10,000.

Protein	Molecular weight	S_{20} ($\times 10^{-13}$ secs^{-1})
Insulin (monomer)	12,000	1.6
RNAase	12,700	1.85
Pepsin	35,500	3.3
Fumarase	204,000	8.51
Actomyosin	3,900,000	12,00

Table R.3. - Sedimentation coefficients and molecular weights of some typical proteins.

f) Determination of molecular weight by osmometry

The plots of reduced osmotic pressure versus concentration for the material in the presence and absence of 0.1 M KCl are shown in Fig. R.4. From this graph:-

$\bar{M}_n$ (KCl absent) = 4.71×10^4

$\bar{M}_n$ (KCl present) = 6.20×10^4

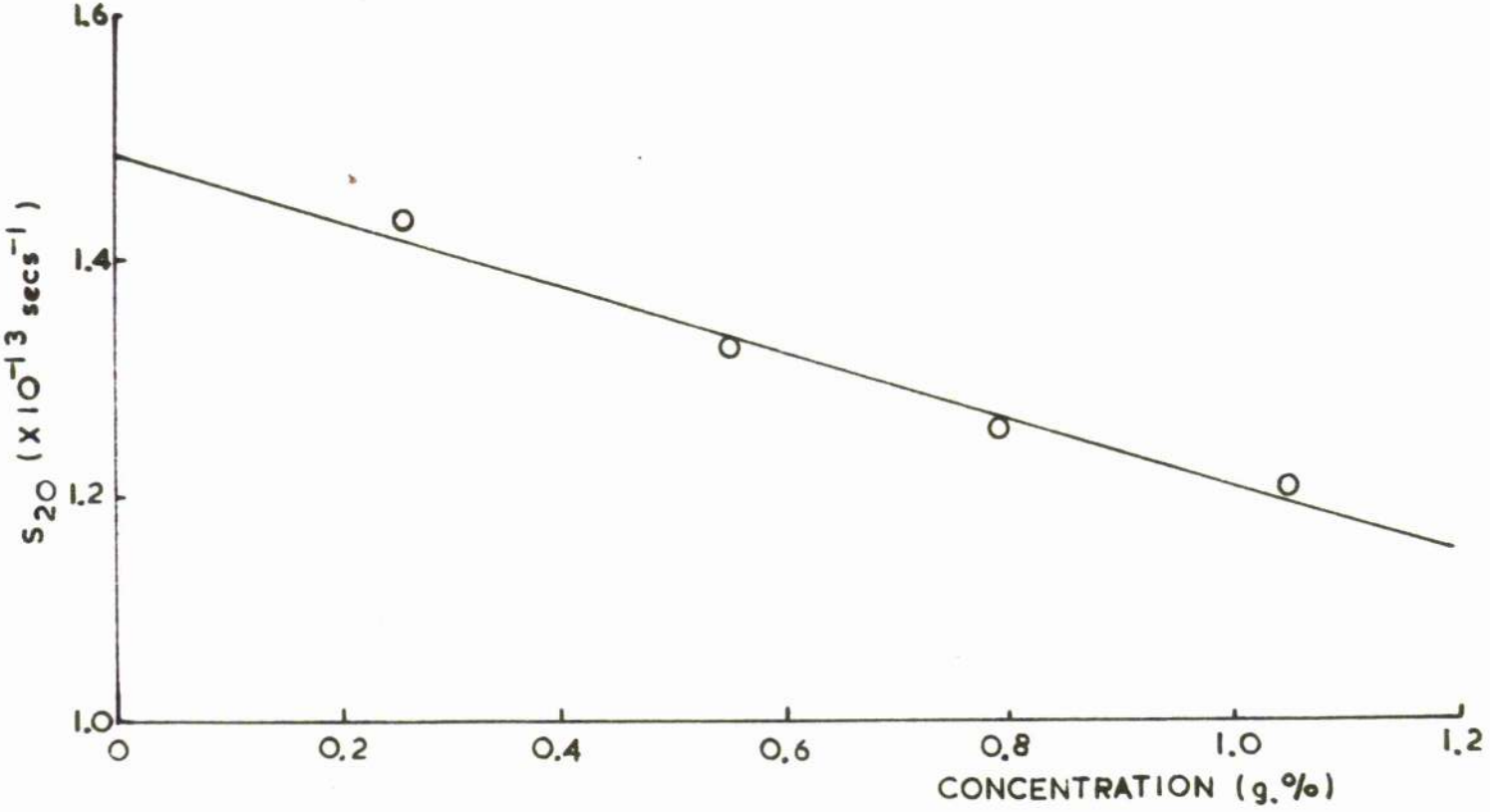


FIG R.2.a, Sedimentation data for Lubrol L solubilised material in 0.1 M KCl

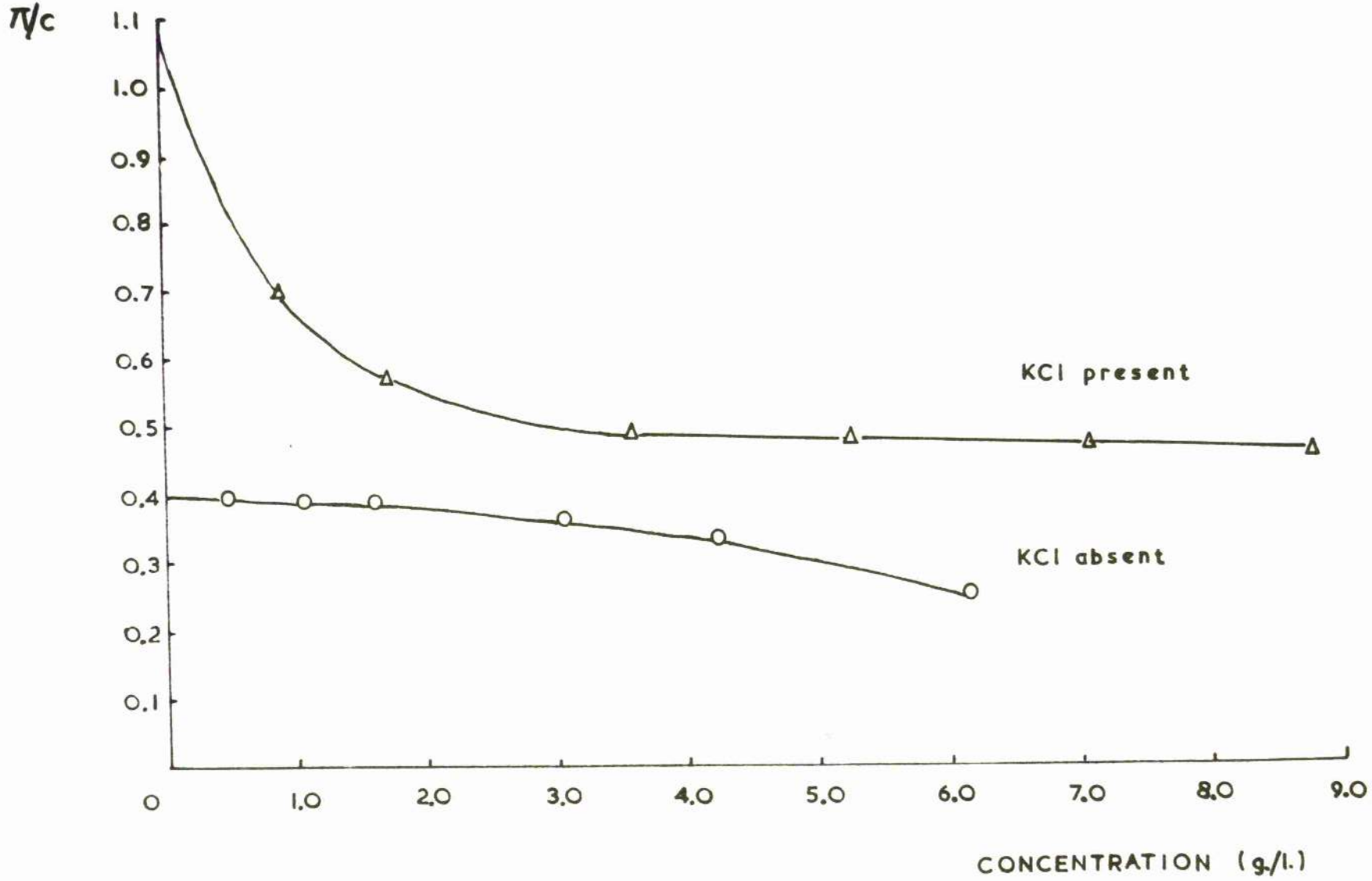


FIG R.4 Reduced osmotic pressure versus concentration for Lubrol L solubilised material in the presence and absence of KCl.

The plot of the data for the material in the absence of KCl is almost linear whilst that for the material in the presence of KCl is decidedly non-linear. Both curves show negative second virial coefficients and this is strongly suggestive of association between macromolecules in solution.

The wide discrepancy between the $\bar{M}_n$ values obtained would indicate that the addition of a salt to a solution of the carotenoid glycoprotein, which was found to still contain detergent, causes the formation of further micelles from any free detergent present or brings about an increase in the size of already existing micelles. The further significance of these observations and the discrepancy between the molecular weight values obtained by osmometry and ultracentrifugation will be dealt with in the discussion.

g) Attempted release of free pigment

The results obtained for the various procedures employed are shown in Table R.4.

C. CHARACTERISATION OF THE POLAR CAROTENOID FRACTION FROM S. FLAV

1. Tests for functional groups, partition ratio and absorption spectra.

The results obtained from these tests were identical for both subfractions and are presented in Table R.5. overleaf. The absorption spectrum recorded for both subfractions and their acetates were identical and are shown in Fig. R.5.

2. Identification of carbohydrate

Of the three solvent systems employed for paper chromatography, n-butanol/acetone/water, 4:5:1 (v/v/v) was found to give

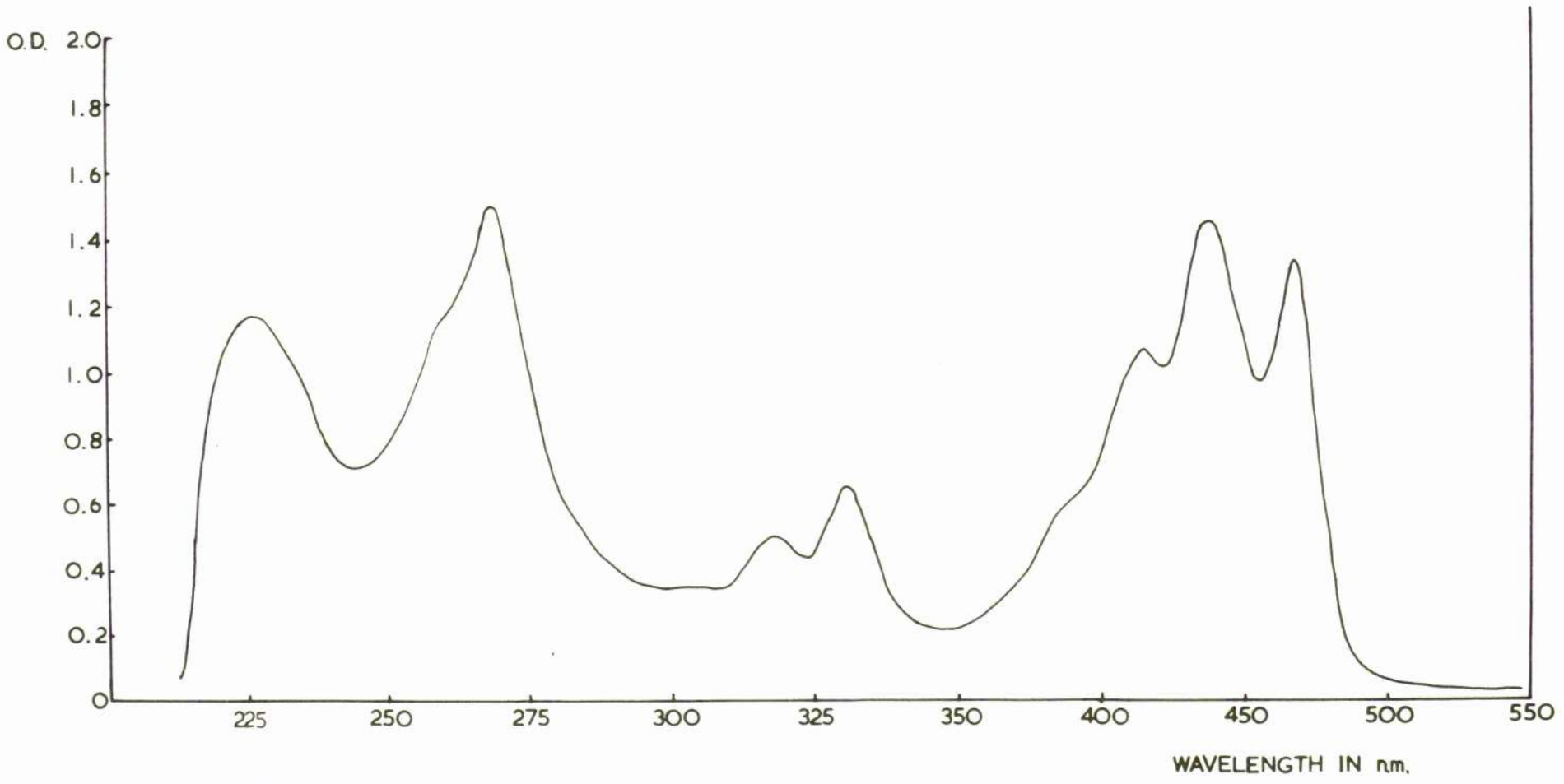


FIG. R.5. Absorption spectrum of polar carotenoid subfraction (i)

inadequate resolution of the standards used and so was not used further.

N HCl hydrolysates of both subfractions were resolvable into 4 anisidine phthalate positive components using each of the other two solvent systems. The $R_{gluc.}$ values of these and of the standards are shown in Table R.6. below.

Method	Result	Comment
(i)	No release	Binding between carotenoid and protein different from that found in invertebrate carotenoproteins where the link is broken by this method, after which 100% of the pigment is extractable into ether, (Cheesman et al., 1967). Acetone here acts as a protein denaturant and thus the integrity of the tertiary structure of the protein moiety is not necessary for the bond.
(ii)	No release	Hydrogen bonding not involved.
(iii)	No release	Hydrogen bonding not involved, fission of any S-S linkages does not aid pigment release.
(iv)	No release	Electrostatic linkages not involved.
(v)	No release	Neither hydrogen bonding nor $\pi-\pi$ electron interactions involved since the thermal energy supplied would have been in excess of that necessary to rupture such bonds.
(vi)	A little pigment released after 6 days	Bonding involving carotenoid very weakly alkali labile.
(vii)	Substantial pigment release	Analysis of pigment released into ether phase showed that it was not free carotenoid but was still associated with carbohydrate, a short peptide and detergent. It is presumed that the effect of papain had been to shorten the peptide/protein moiety to a length which made a portion of the material preferentially soluble in ether.

Table R.4. - Attempts to release free carotenoid from the Lubrol L solubilised carotenoid glycoprotein.

Test	Result	Comment
Acetylation of primary/secondary OH.	Positive, a minimum of 5 intermediates detectable, excluding starting material and final acetate	At least 6 hydroxyls (primary/secondary) acetylated. After complete acetylation, subfractions still relatively polar suggesting presence of additional polar functions other than primary/secondary hydroxyls.
Trimethylsilylation of tertiary OH.	Negative	
Methylation of COOH.	Positive	Amino acid analysis indicated that COOH groups being methylated were probably those of aspartic and glutamic acids.
Partition ratio	100% into methanol	Highly polar compound
U.V. and visible absorption.	Maxima at 220,264, 313,326,391,415, 437 and 467 nm. for both subfractions and final acetates.	Maximum at 220 due to peptide bond, maxima in visible region due to carotenoid and not significantly altered from those of the free pigments, thus association of carotenoid with other molecules does not involve any delocalisation of the conjugated chromophore of double bonds by e.g. electrostatic interaction.
I.R. spectrum	Spectrum very complex, but no cis peak	Carotenoid in all-trans form. Complexity of spectrum due to presence of carbohydrate and amino acids in the compound.

Table R.5. - Some analytical results obtained for subfractions (i) and (iii) of the polar carotenoid from S. flava.

Solvent System.	$R_{glucose}$ Values					
	Gluc	Gal	Man	Rib	Subfraction (i)	Subfracti (iii)
BuOH/HAc/H_2O	1.00	0.87	1.21	1.62	0.99, 0.55,	0.99, 0.5
6:1:2					0.39, 0.12	0.33, 0.1
BuOH/Pyr/H_2O	1.00	0.91	1.16	1.34	0.99, 0.54	0.98, 0.5
6:4:3					0.37, 0.11.	0.30, 0.1

Table R.6. $R_{glucose}$ values of standards and unknowns from hydrolysates of subfractions (i) and (iii).

As can be seen from their $R_{gluc.}$ values, 3 of the anisidi phthalate positive components may well be common to both subfractions. That with the highest $R_{gluc.}$ was found, in the case of bo subfractions to co-chromatograph with glucose in the butanol/pyridine/water system. This component was also present in much greater concentration than any of the other three.

When duplicate chromatograms were stained with ninhydrin, 8 components were visible for each subfraction, 4 of which had th same R_f values as those staining for reducing sugar and 4 of whic had even lower R_f values than these.

3. Resolution of carbohydrate and peptide/glycopeptide

For both subfractions, duplicate two-dimensional paper chromatograms showed that the anisidine phthalate positive component which co-chromatographed with glucose was in fact free glucose and not associated with ninhydrin positive material. Th remaining 3 components still stained with both anisidine phthalat and ninhydrin so that it is presumed that these are glycopeptides

(R_fs too low to be amino sugars).

4. Resolution of peptides/glycopeptides

The two-dimensional T.L.C. system used resolved the N HCl hydrolysate from subfraction (i) into 8 ninhydrin positive components, as did paper chromatography but for subfraction (iii) 10 spots were visible after ninhydrin staining. Accurately traced drawings of the two chromatograms are shown in Figs. R.6. and R.7. The cclour of each spot after staining with ninhydrin is denoted according to the following code: R = Red, O = Orange, P = purple. When compared with published R_f values for amino acids chromatographed in the same system, it was apparent that none of the components of either hydrolysates was an amino acid. This is to be expected since hydrolysis with N HCl under the conditions used would almost certainly lead to incomplete hydrolysis and result in the production of peptides or glycopeptides.

From the chromatograms it can be seen that the peptides liberated by N acid hydrolysis are not the same for the two subfractions. This indicated that the original peptide moiety from which the hydrolysis products are derived has a different composition in each case. An alternative explanation might be that a minor difference in either time or temperature of hydrolysis could result in differences in the number and composition of the products. However, subsequent amino acid analysis proved the former assumption correct, although the possible influence of variations in hydrolysis conditions cannot be discounted.

5. Linkage of carotenoid to glucose

Anisidine phthalate reacts with the free reducing groups

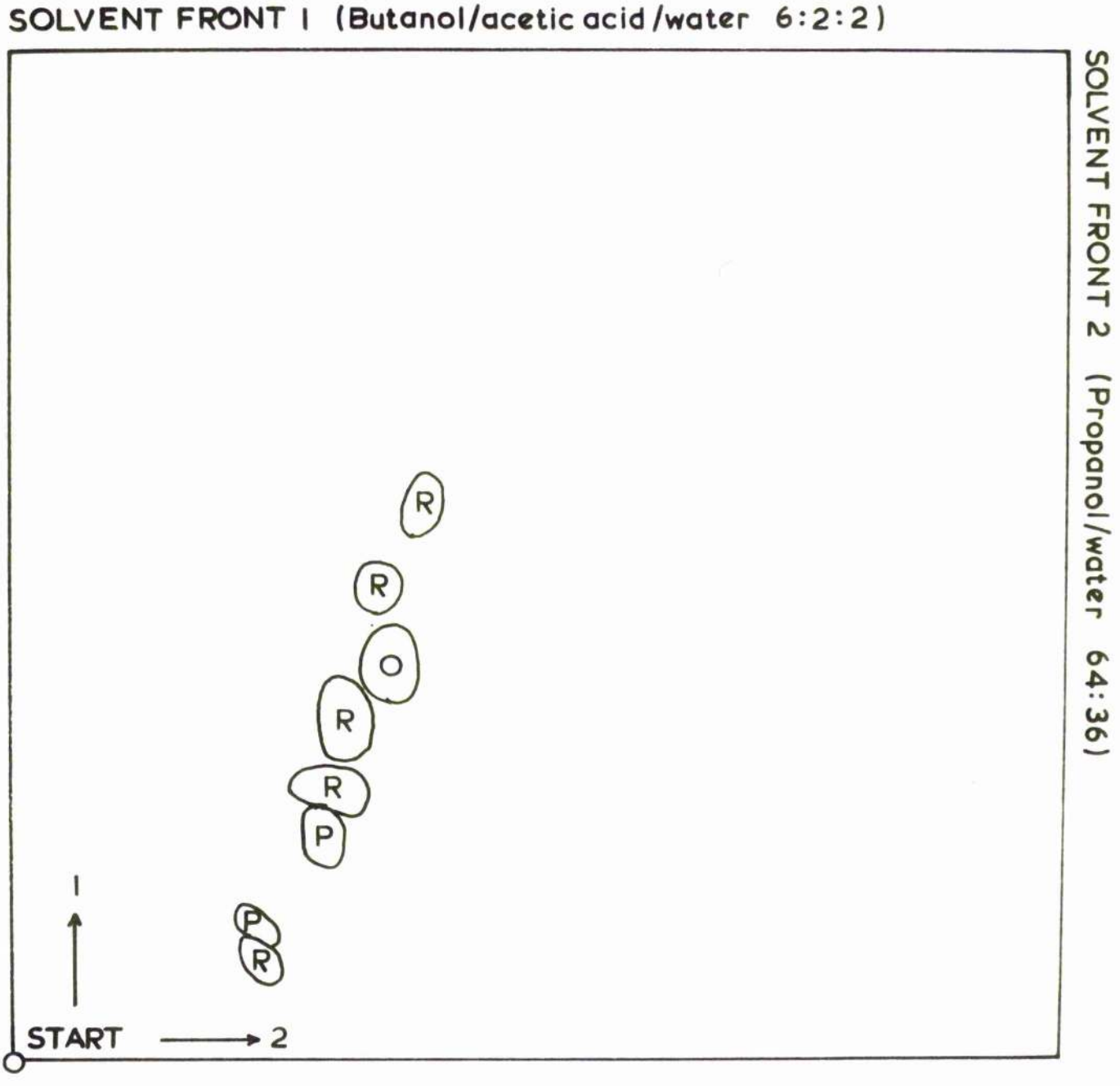


FIG. R.6. Thin-layer chromatogram of peptides from subfraction (i)

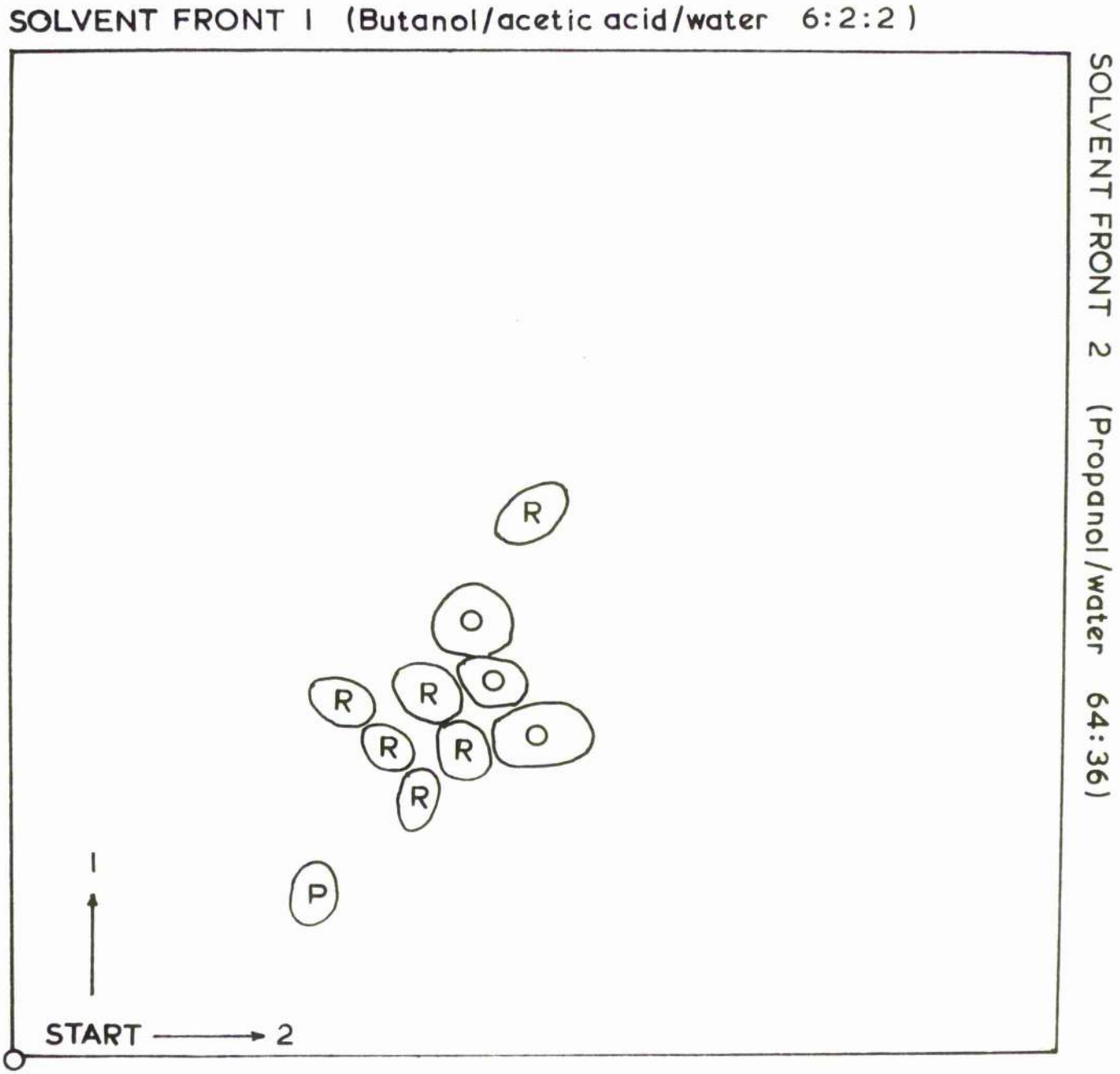


FIG R7 Thin-layer chromatogram of peptides from subfraction (iii)

of sugars only and, since the subfractions before hydrolysis did not react with the stain, but the hydrolysates did, it is inferred that the carotenoid is linked glycosidically to glucose in this material. As indicated in the introduction, the occurrence of carotenoid glycosides in nature is now known to be widespread. Such a linkage would certainly be split by N acid hydrolysis as reported here.

6. Amino acid composition

The results of amino acid analysis are shown below in Table R.7. where amounts of each amino acid are expressed as moles

Amino acid	Subfraction (i)	Subfraction (iii)
Aspartic acid	1.3	2.0
Threonine	1.2	1.2
Serine	3.0	3.0
Glutamic acid	3.2	5.0
Glycine	6.1	7.1
Alanine	2.1	3.0
Valine	1.0	2.0
Isoleucine	0.8	1.8
Leucine	1.0	2.1
Tyrosine	0.8	1.0
Phenylalanine	0.8	1.1
Ornithine	1.4	2.8
Lysine	1.0	2.0
Histidine	0.9	1.1

Table R.7. - Amino acid composition of subfractions (i) and (iii)

Serine, glutamic acid and glycine are the most abundant amino acids in both subfractions and the sulphur-containing amino acids are totally absent. It is worthy of note that all the

amino acids, with the possible exception of ornithine, some or all of which may be derived from arginine during hydrolysis, are present in integral molar ratios. This is indicative of a repeating unit within the peptide from which the amino acids are derived.

7. Quantitative chemical composition

The carotenoid, glucose and total amino acid content of each subfraction are shown below in Table R.8.

Subfraction	% Carotenoid	% Glucose	% Amino Acid
(i)	42	23	24
(iii)	40	24	27

Table R.8. - Chemical composition of subfractions (i) and (iii).

D. HOMOGENEITY AND PROPERTIES OF A WATER-SOLUBLE CAROTENOID GLYCOPEPTIDE FROM S. MORRHUAE.

1. Homogeneity

a) Ultracentrifugation

The sedimentation behaviour of the material indicated that it consisted of a monodisperse system of molecules, if not a single molecular species. A single, perfectly symmetrical peak was observed resembling that shown earlier in Fig. R.2. for the detergent solubilised material from S. flava. The rates of migration of the two peaks were also very similar, indicating that the molecules may be of comparable size.

b) Sephadex gel-filtration

No resolution of the material was obtained, as judged by orange colour and absorbance at 275 nm., on any grade of Sephade

used (G25, 50, 75 & 100).

c) Agarose column chromatography

The elution pattern obtained for the material from the Biogel A15 column is shown in Fig.R.8. Only a portion of the elution diagram is shown since all fractions before tube 60 (90 mls effluent) and after tube 125 (187.5 mls) showed zero absorbance at 276 nm.

Tubes 80 - 90 (120 - 135 mls) contained orange fractions, the majority of the colour being concentrated in tubes 83-87. The symmetry of the single peak obtained again indicates that the material is homogeneous.

d) Disc-gel electrophoresis

The material migrated as a single compact band, under the conditions of the experiment, at approximately the same rate as the serum albumin standard used.

2. Properties

a) Molecular weight

A molecular weight for the material of approximately 8,000 - 9,000 is suggested by the following data:-

(i) slow diffusion out of visking dialysis tubing (molecular weight exclusion limit about 10,000).

(ii) The material was eluted in the void volume from the Sephadex G25 column (molecular weight fractionation range 100 - 5,000), but was retained to an increasing extent on the columns of G50 (500 - 10,000), G75 (3,000 - 70,000) and G100 (4,000 - 150,000).

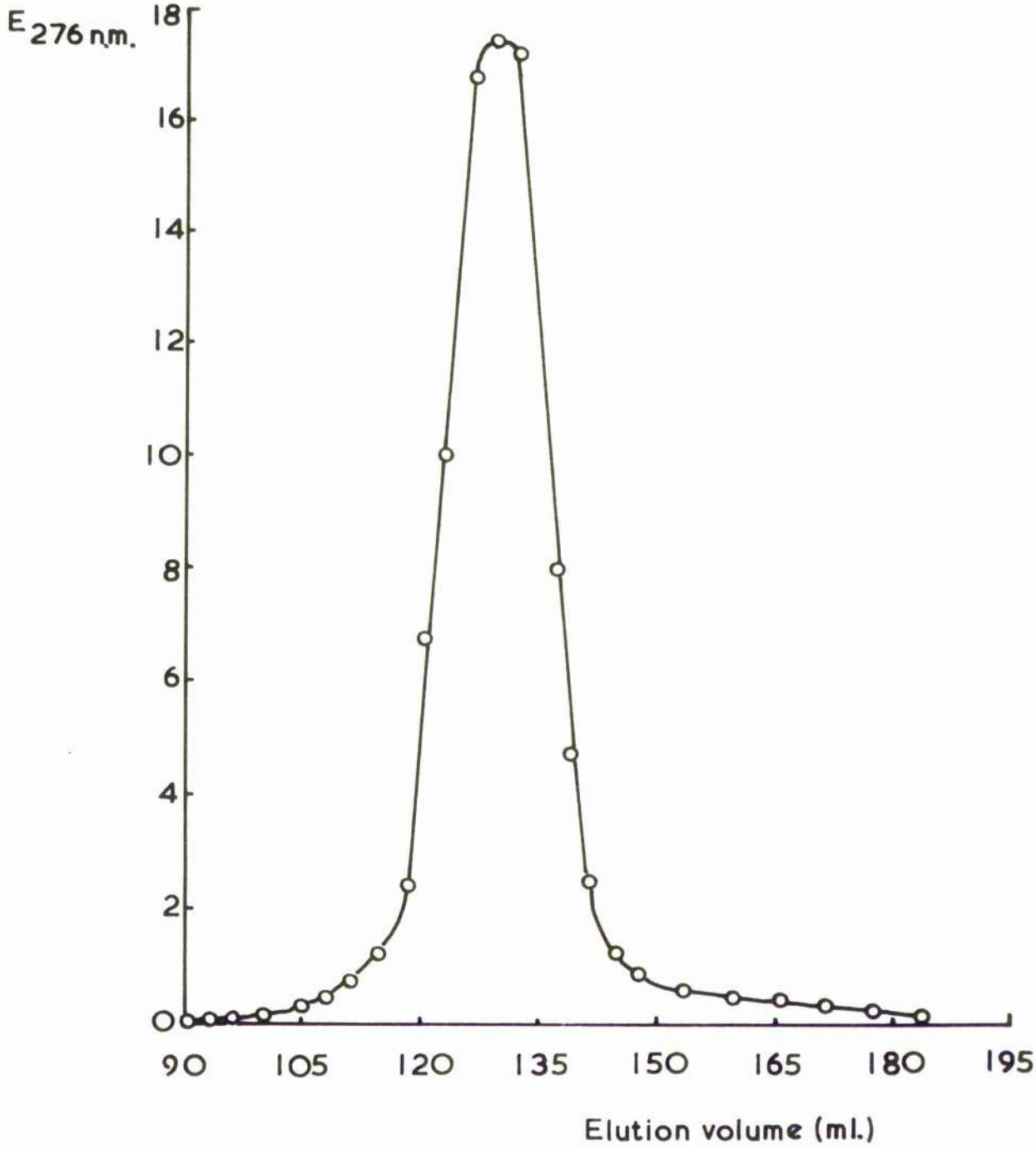


FIG.R.8. Elution diagram obtained from Biogel A 15 column for a carotenoid glycopeptide from Sarcina morrhuae.

(iii) The position of elution from the Biogel A15 column enabled the calculation of a Stoke's radius for the material of 5 Å. Since this was outwith the molecular weight range of the standard graph, the only interpretation which can be made from this value is that the molecular weight is less than 10,000.

b) Absorption spectrum - the presence of carotenoid

Maxima were recorded at 237 and 276 nm. but their intensity was such that no maxima could be distinguished in the visible range. The presence of carotenoid in the material can nevertheless be deduced since:-

(i) the material is red-orange in colour and the bacterium synthesises red-orange free pigment,

(ii) the material gives a positive Carr-Price reaction with $SbCl_3$ in chloroform (Morton, 1942).

c) Amino acid analysis

The amino acid composition is shown in Table R.9. below. Results are expressed in moles per cent.

Amino acid	Moles %	Amino acid	Moles %
Aspartic acid	13.3	Isoleucine	5.6
Threonine	4.1	Leucine	8.9
Serine	2.7	Tyrosine	2.6
Glutamic acid	14.0	Phenylalanine	3.4
Glycine	13.0	Lysine	2.3
Alanine	11.1	Histidine	2.6
Valine	9.1	Ornithine + arginine	5.7
Methionine	1.6		

Table R.9. - Amino acid composition of a carotenoid glycopeptide from S. morrhuae.

The two amino acid residues present in the greatest concentration are aspartic and glutamic acids and the small ammon peak recorded for this analysis would suggest that little, if any of either is present in its respective amide form. Together with glycine and alanine, these acidic amino acids account for more than 50% of the total amino acids in the material. With the exception of a small amount of methionine, the sulphur-containing amino acids are again absent.

Small unidentified peaks were also observed between the following components in the elution sequence from the analyser:-

(i) glutamic acid and glycine

(ii) phenylalanine and ammonia

(iii) methionine and isoleucine

(iv) lysine and histidine

d) Identification of carbohydrate

Both solvent systems resolved the hydrolysate into 2 anisidine phthalate positive components. One, which co-chromatographed with glucose in both solvent systems was present in much greater concentration than the other, which possessed a much lower R_f value. $R_{gluc.}$ values for the standards in both the solvent systems have already been reported elsewhere in this section.

Spraying of duplicate chromatograms with ninhydrin showed the presence of 12 ninhydrin positive components, all of low R_f value in comparison with the free monosaccharide standards. One of these components had an identical R_f to that of the minor spot

which stained for reducing sugar, and this is again presumed to be a glycopeptide. The remaining ninhydrin positive components are probably small peptides derived from the peptide moiety of the material by the acid hydrolysis.

e) Linkage between carotenoid and glucose

As was suggested for the polar carotenoid subfractions from S. flava, the linkage between carotenoid and glucose in this material is in all probability glycosidic since the unhydrolysed material does not stain with anisidine phthalate whereas the N HCl hydrolysate is anisidine phthalate positive.

f) Attempted release of free pigment by hydrolysis

After treatment with 0.1 N HCl and ether extraction, the ether phase was faintly yellow, indicating that some pigment had been released by this method. However, spectrophotometric examination of the concentrated ether extract revealed no maxima in the visible region which suggests that although some carotenoid may have been released, it was immediately degraded under the acidic conditions.

Enzymic hydrolysis with papain and with almond emulsin did not cause the release of any pigment into the ether phase under the conditions of digestion employed.

Attempts by other workers (Hertzberg et al., 1967) to hydrolyse the glycosidic bond in the carotenyl tertiary-D-glucoside phleixanthophyll (see introduction) using almond emulsin were also unsuccessful, although release of free pigment was achieved by treatment with 0.02 N HCl in chloroform. However,

as indicated above, treatment of this material from S. morrhuae with HCl of less concentration than 0.1 N did not result in the release of carotenoid.

g) Quantitative chemical composition

The ash, protein, carbohydrate and carotenoid contents of the material are summarised in Table R.10. below.

	% by weight	Molar ratio
Ash[1]	17.5	-
Protein[2]	59.7	26.2[3]
Carbohydrate	7.7	2.0[4]
Carotenoid	15.1[5]	0.95[6]

Notes:- 1. Calculated as chloride, since this is known to be the main intracellular anion in S. morrhuae (Christian et a 1962).

2. Estimation of protein by Folin-Lowry, Moore et al., (1954) and amino acid analysis gave good agreement between results.

3. Calculated using the mean molecular weight of the amino acids shown to be present by autoanalysis (Table R.9.).

4. Calculated as glucose.

5. Estimated by difference, since attempts to free pigment unsuccessful.

6. Calculated as bacterioruberin since this has been shown to be the only free pigment synthesised by the organism

Table R.10. - Chemical composition of a carotenoid glycoprotein from S. morrhuae.

E. CHEMICAL ANALYSIS OF MEMBRANES FROM S. FLAVA

1. Yield of membrane fraction

The percentages of the dry weight of the three ages of bacterial cell constituted by the total membrane fractions are shown in Table R.11. below. Each value is the mean of two determinations.

Age of membrane	% Dry Weight
24 hrs.	49.6
67 hrs.	36.8
91 hrs.	34.5

Table R.11. - Yield of total membrane fraction in relation to bacterial dry weight.

2. Moisture and ash content

The results of these determinations are shown below in Table R.12.

Age of membrane	% Moisture	% Ash
24 hrs.	1.4	8.2
57 hrs.	1.4	7.2
91 hrs.	1.8	7.6

Table R.12. - Variation in moisture and ash content with age.

These values were used to express the protein, lipid, carbohydrate and RNA contents of the membrane preparations as percentages of total organic material.

3. Carbohydrate content

The results of carbohydrate estimation by both methods are shown below in Table R.13. Each value is expressed as a percentage of the total organic material and is the mean of three

determinations, each performed in duplicate.

Age of Membrane	% Carbohydrate (Phenol/H_2SO_4)	% Carbohydrate (Anthrone)
24 hrs.	13.8	6.3
57 hrs.	14.5	6.7
91 hrs.	14.3	7.3

Table R.13. - Variation in carbohydrate content with age.

The results obtained by the phenol/H_2SO_4 method show a carbohydrate content which is approximately double that as determined by the anthrone method. Both methods gave reproducible results for duplicate samples in the same determination and also for different determinations.

As mentioned previously, the phenol/H_2SO_4 method would seem to be the more reliable for analysis of membrane material since many compounds are known to interfere with the anthrone method. It should be said however, that where a colorimetric method is liable to interference from a contaminating substance, the effect is usually for the observed optical densities to be elevated rather than depressed.

Other workers (Gilby et al., 1958; Salton, 1967c; Ghosh et al., 1968) have used the anthrone method for the estimation of membrane carbohydrate, but in these cases an alternative method was not used to confirm the values obtained.

Allowing for experimental error, (1 - 2%) there would appear to be little variation in total carbohydrate composition of the membrane with age, whichever method of estimation is used.

4. Lipid content

The results are presented in Table R.14. below. Values

are expressed as percentages of total organic material, and each is the mean of two determinations. The figures in parentheses represent the percentage of the total lipid constituted by each fraction.

Age of Membrane	Free Lipid	Acid Hydrolysate	Base Hydrolysate	Total Lipid
24 hrs.	8.0 (40.0)	9.1 (45.8)	2.8 (14.2)	19.9
57 hrs.	7.6 (38.0)	8.0 (40.8)	4.2 (21.2)	19.8
91 hrs.	5.4 (37.2)	6.1 (42.1)	3.2 (20.7)	14.7

Table R.14. - Variation in lipid content with age

The total lipid contents of the 24 and 57 hour preparations are very nearly identical whereas the value is considerably (26%) less for the 91 hour membranes. It is perhaps significant that the percentage contribution to the total lipid made by the free and acid hydrolysed lipid together decreases with age, whilst the contribution from the base hydrolysed lipid shows an increase with age, especially between 24 and 57 hours.

5. Amino acid composition

The results of amino acid analysis are shown below in Table R.15. Values are expressed as moles per cent and each is the mean of two analyses, performed on separate hydrolysates.

In the hydrolysates from all three membrane preparations 2 unidentified components were detected which occupied the following positions on the analyser read-out:-

(i) between alanine and valine

(ii) between phenylalanine and ammonia

(i) may possibly be glucosamine, and was a fairly sizeable component

(ii) may be ethanolamine and had a small peak area.

	Age of Membrane		
Amino acid	24 hrs.	57 hrs.	91 hrs.
Aspartic acid	7.5	9.8	7.8
Threonine	4.6	5.0	4.6
Serine	3.9	4.3	4.3
Glutamic acid	12.4	13.0	12.6
Proline	3.4	4.4	4.5
Glycine	13.0	10.7	12.2
Alanine	16.0	14.8	16.1
Valine	8.3	8.1	8.0
Cysteine	0.3	0.2	0.1
Methionine	1.4	1.1	1.5
Isoleucine	3.1	3.0	2.9
Leucine	7.2	7.1	7.2
Tyrosine	1.8	1.7	1.7
Phenylalanine	2.5	2.6	2.5
Lysine	7.5	7.3	6.8
Histidine	2.0	2.0	2.0
Arginine	5.3	5.0	5.1

Table R.15. - Variation in amino acid composition with age

The most abundant amino acids in all three ages of membrane are glutamic acid, glycine and alanine, and once again it can be seen that there is a predominance of the acidic and apolar residues in relation to the basic and sulphur-containing amino acids.

The molar ratios of the individual amino acids show little variation with age, the only significant variations being an increase in aspartic acid, and a decrease in glycine and alanine contents for the 57 hour preparation.

The total protein content of the membranes, as determine from the amino acid analyses is shown below in Table R.16. where results are expressed as percentages of total organic material.

Age of Membrane	% Protein
24 hrs.	63.6
57 hrs.	56.1
91 hrs.	51.2

Table R.16. - Variation in total protein content with age

Despite the constancy of the molar ratios of the amino acids, the overall protein content decreases markedly with age. This observation is particularly surprising for the 91 hour preparation since the total lipid content of these membranes is also less than that for the other preparations, whilst the carbohydrate content shows only a small increase by comparison.

6. Total phosphorus content

The results are the mean of two separate determinations, each performed in triplicate, and are tabulated below in Table R.17.

Age of Membrane	% P
24 hrs.	1.2
57 hrs.	0.8
91 hrs.	0.7

Table R.17. - Variation in total P content with age.

The membranes from the 24 hour culture, which was still in late exponential phase of growth when harvested, contain 50% more phosphorus than membranes prepared from cells in stationary phase.

7. RNA content

Results are the mean of two determinations, each perform in duplicate, and are shown in Table R.18. below.

Age of Membrane	% RNA
24 hrs.	2.9
57 hrs.	1.9
91 hrs.	2.3

Table R.18. - Variation in RNA content with age

8. Summary of quantitative chemical analyses

The overall chemical composition of the three ages of membrane is presented overleaf in Table R.19.

9. Identification of membrane carbohydrates

a) Paper chromatography

The solvent system n-butanol/pyridine/water, 6:4:3 (v/v/v) gave good resolution of standards and unknowns. The $R_{gluc.}$ values for the standards are presented in Table R.20. below.

Sugar	$R_{glucose}$	Colour of spot
Glucuronic acid	0.21	Red-brown
Galactosamine hydrochloride	0.72	Brown
Glucosamine hydrochloride	0.76	Brown

Age of Membrane	Moisture[1]	Ash[1]	Carbohydrate (phenol/H_2SO_4)[2]	Carbohydrate (anthrone)[2]	Total Lipid[2]	Total P[1]	Protein[2]	RNA[2]
24 hrs.	1.4	8.2	13.8	6.3	19.9	1.2	63.6	2.9
57 hrs.	1.4	7.2	14.5	6.7	19.8	0.8	56.1	1.9
91 hrs.	1.8	7.6	14.3	7.3	14.7	0.7	51.2	2.3

Notes:- 1. Expressed as percentage of total dry weight

2. Expressed as percentage of total organic material

Table R.19. - Quantitative Chemical Composition of Different Ages of Membrane.

Mannosamine hydrochloride	0.88	Brown
Galactose	0.91	Brown
Glucose	1.00	Brown
Arabinose	1.05	Rose-red
Mannose	1.16	Brown
N-acetyl glucosamine	1.21	Brown
Ribose	1.34	Rose-red

Table R.20. - $R_{gluc.}$ values of standard carbohydrates for the descending solvent system : n-butanol/pyridine/wat 6:4:3 (v/v/v).

All three membrane hydrolysates contained the same anisidine phthalate positive components, and their $R_{gluc.}$ values and tentative identification are presented below in Table R.21.

Component	$R_{glucose}$	Colour	Identity
1	0.22	Brown	Glycopeptide (?)
2	0.73	Brown	Galactosamine
3	0.99	Brown	Glucose
4	1.14	Brown	Mannose
5	1.35	Rose-red	Ribose

Table R.21. - $R_{gluc.}$ values of components from membrane hydrolysates for the descending solvent system : n-butanol pyridine/water, 6:4:3 (v/v/v).

From the relative size and intensities of the spots, glucose and mannose appeared to be the components present in the highest concentration. Component 1, although possessing the same $R_{gluc.}$ as glucuronic acid did not give the red-brown colou

typical of authentic glucuronic acid, when sprayed with anisidin phthalate, and is in all probability a glycopeptide, although th was not investigated further as it constituted only a very minor proportion of the total anisidine phthalate staining material.

b) Gas-liquid chromatography

The retention distances of the trimethylsilyl derivative of the standard sugars, relative to that of ∝-methyl-D-glucosid are shown below in Table R.22. Figures in parentheses represen peaks corresponding to the β-anomers of the sugars, where presen In the case of fucose, a γ-peak is also apparent.

Carbohydrate	R_{α} ∝-methyl-D-glucoside
Arabinose	0.339 (0.383)
Rhamnose	0.356 (0.490)
Ribose	0.395 (0.428)
Fucose	0.442 (0.523, 0.375)
Mannose	0.821 (1.126)
Glucose	1.073 (1.367)
Galactose	1.125 (1.126)
N-acetyl glucosamine	1.712

Table R.22. - R_{α} ∝-methyl glucoside values for trimethylsilyl ethers of standard carbohydrates on G.L.C.

As shown by paper chromatography, all three ages of membrane were found to contain the same carbohydrate components by G.L.C. A typical gas-liquid chromatogram for the 57 hour membrane hydrolysate is shown in Fig. R.9. Peaks marked ' ?, did not have retention distances corresponding to any of the

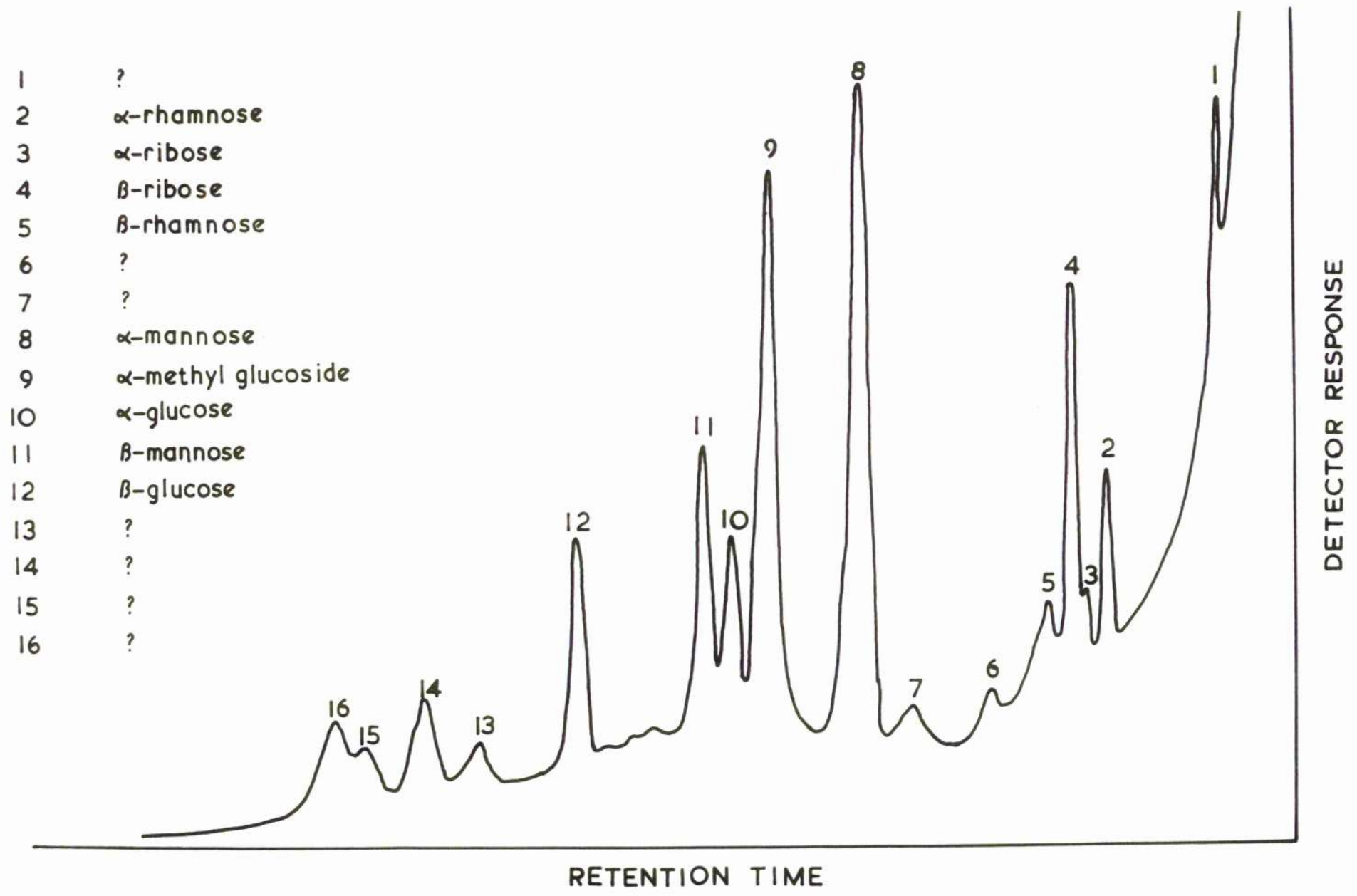


FIG.R9. Gas-liquid chromatogram of T.M.S. derivatives of S. flava membrane carbohydrates.

standards used, and those with rather long retention times may well be incompletely hydrolysed oligosaccharides.

Once components of the hydrolysates had been tentatively identified from their $R_{\alpha\text{-methyl glucoside}}$ values, their identities were confirmed by co-chromatography with an authentic standard of each suspected sugar in turn.

In this way, the following carbohydrates were identified in all three membrane hydrolysates:-

(i) ribose

(ii) rhamnose

(iii) mannose

(iv) glucose

Thus, in addition to confirming the results of paper chromatography, G.L.C. analysis also established the presence of rhamnose, which was not detected by the former technique. As estimated by peak area, these four sugars were present in the following descending order of concentration: mannose glucose ribose rhamnose. The very small proportion of rhamnose in relation to the remaining monosaccharides probably accounts for the fact that it was not detected by the less sensitive techniqu of paper chromatography.

10. Fatty acid composition

A typical gas-liquid chromatogram showing the fatty acid methyl esters present in the base hydrolysed lipid fraction from 91 hr. membranes is shown in Fig. R.10. The complete results o the G.L.C. analysis are presented in Table R.23. and from these

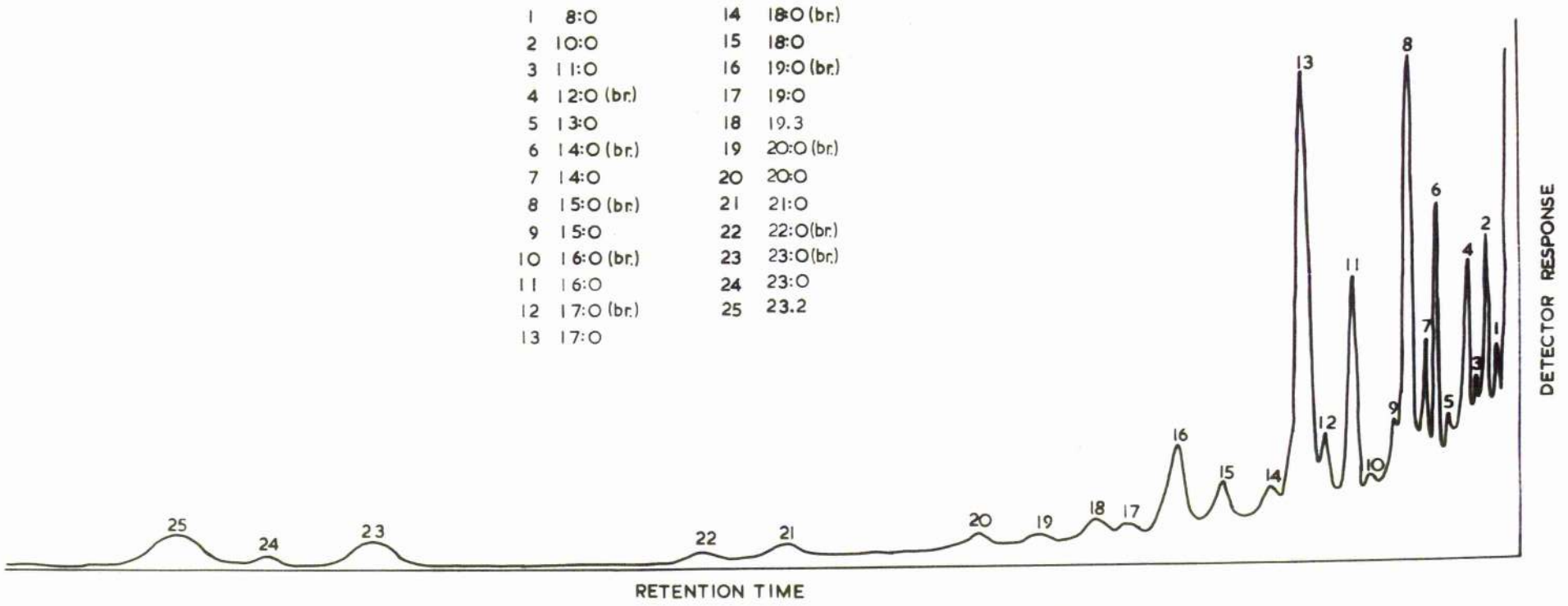


FIG. R.10. Gas-liquid chromatogram of fatty acid methyl esters from base hydrolysed lipid fraction of 91 hour membranes.

and the figure it can be seen that the membrane lipids contain a wide range of fatty acids, and that the fatty acid composition varies markedly with the age of the bacterial culture.

The major fatty acid of the free lipid fractions from all three ages of membrane is a fully saturated, branched chain C_{15} compound whereas the chief component of both the bound lipid fractions in all cases is the fully saturated, straight chain, C_{17} acid.

The proportion of this C_{17} acid in both the bound lipid fractions remains virtually constant with age whilst the proportion of the branched C_{15} component of the free lipid decreases markedly from 24 to 57 hrs. increasing again slightly at 91 hrs. Furthermore, this marked decrease at 57 hours is accompanied by a concommitant increase in the proportion of the straight chain C_{17} acid.

The amount of unsaturated fatty acids present is low in the fractions examined.

11. Examination of the non-saponifiable fractions for the presence of steroid.

a) Thin-layer chromatography

T.L.C. in the system used by Avigan et al. (1963) for the separation of steroids, and subsequent staining with $SbCl_3$/chloroform produced the chromatogram shown in Fig. R.11. All 6 non-saponifiable lipid fractions contained the same component which gave the pink colour with $SbCl_3$, characteristic of steroids, and which possessed a similar R_f to that of the cholesterol standard,

as can be seen in the figure. A large amount of pigment and other hydrocarbon material was also present and this can be seen at the solvent front.

It was further found, for each non-saponifiable fraction, that a white precipitate was formed on addition of digitonin, which, after purification according to the method of Sperry and Webb (1950), gave a positive Liebermann-Burchardt test. These observations suggested that the non-saponifiable fractions contained a sterol and that the sterol might well be cholesterol.

b) Gas-liquid chromatography

It was found by G.L.C. analysis that all the unsaponifiab fractions contained between 20 and 30 components. Some of these are almost certainly long chain non-isoprenoid hydrocarbons which have been shown to make up a large proportion of the lipids of th related species S. lutea (Albro et al., 1969) and would certainly be extracted in this fraction.

However, none of these components had a retention time which even approximated to that of the cholesterol standard, so that if, as is suggested by the T.L.C. data digitonide formation and positive Lieberman-Burchardt test, a sterol is present in S. flava membrane lipids, then it seems unlikely that it is cholesterol. Work is in progress to further characterise this compound.

Fatty acid	24 h.	57 h.	91 h.	24 h.	57 h.	91 h.	24 h.	57 h.	91 h.
Br. 8:0	-	-	-	-	Tr.	-	-	-	-
8:0	Tr.	Tr.	Tr.	1.8	Tr.	-	1.2	Tr.	Tr.
8:1	-	Tr.	-	-	Tr.	Tr.	-	-	-
Br. 9:0	-	-	-	6.7	-	-	-	-	-
9:0	Tr.	-	Tr.	-	-	-	-	-	-
10:0	3.2	0.8	Tr.	1.8	-	1.5	4.4	1.4	1.2
10:1	-	3.1	-	-	1.6	-	-	-	-
Br. 11:0	2.3	-	Tr.	-	-	-	-	-	-
11:0	-	-	-	2.0	1.7	1.0	1.3	Tr.	Tr.
11:1	Tr.	Tr.	-	-	-	-	-	-	-
(11:1)	-	-	-	4.3	-	-	-	-	-
Br. 12:0	Tr.	2.1	-	-	-	5.0	3.3	-	2.1
(12.3)	-	-	10.1	-	15.6	-	-	-	-
Br. 13:0	4.9	3.2	3.6	-	-	-	-	-	-
13:0	-	-	-	2.2	1.0	1.6	1.0	Tr.	Tr.
Br. 14:0	4.1	2.3	1.6	1.0	Tr.	Tr.	10.8	8.3	4.7
14:0	1.0	1.8	2.3	3.7	4.4	3.1	4.1	2.1	2.7
Br. 15:0	46.3	20.6	37.6	8.6	1.5	11.4	3.2	11.4	20.0
15:0	-	-	-	2.6	4.2	-	1.2	1.2	1.5
(15.2)	-	-	-	-	-	-	1.1	-	-
Br. 16.0	1.1	Tr.	Tr.	1.0	1.6	1.0	Tr.	Tr.	Tr.
16:0	5.2	9.8	4.1	-	11.7	6.9	7.3	5.7	7.9
Br. 17:0	Tr.	2.4	3.4	14.4	3.3	5.1	1.6	13.5	2.7
17:0	2.9	15.0	8.0	22.0	22.4	24.2	10.7	20.9	20.8
Br. 18.0	-	Tr.	-	1.7	-	4.7	1.3	1.1	1.0
18:0	4.4	9.3	3.0	6.9	3.1	2.3	3.8	2.2	2.6
(18.2)	-	-	-	-	-	-	1.2	-	-
Br. 19:0	Tr.	3.0	4.3	10.0	2.4	3.1	2.4	3.9	6.3
19:0	-	1.4	1.7	3.0	2.1	2.1	1.1	Tr.	Tr.
(19.3)	-	-	-	-	-	-	1.2	1.2	1.7
Br. 20:0	-	Tr.	-	1.7	-	1.9	1.6	Tr.	Tr.
20:0	-	2.0	1.4	3.1	1.9	3.9	1.8	1.1	Tr.
(20.3)	3.6	-	-	-	-	-	-	-	-
Br. 21:0	5.0	-	Tr.	-	-	Tr.	Tr.	-	-
21:0	4.5	-	6.1	-	-	-	4.2	2.2	2.4
(21.2)	6.7	5.0	-	-	-	-	-	-	-
Br. 22:0	1.1	-	-	1.2	10.7	7.8	1.9	-	Tr.
22:0	-	-	-	-	-	4.5	-	-	-
(22.3)	1.7	1.4	1.4	-	-	-	-	-	-
Br. 23:0	Tr.	4.4	3.8	Tr.	6.5	5.2	10.9	3.7	6.5
23:0	-	-	-	-	-	-	3.0	3.0	1.5
(23.2)	-	6.1	-	-	-	-	3.0	11.7	10.7
Br. 24:0	-	2.0	2.4	Tr.	-	2.4	-	-	-
Recovery	98.0	95.7	94.8	99.7	97.7	98.7	07.7	94.6	96.3

Notes:-

(i) Results are expressed as % total fatty acids and all components present in amounts less than 1% are denoted as Tr. = Trace.

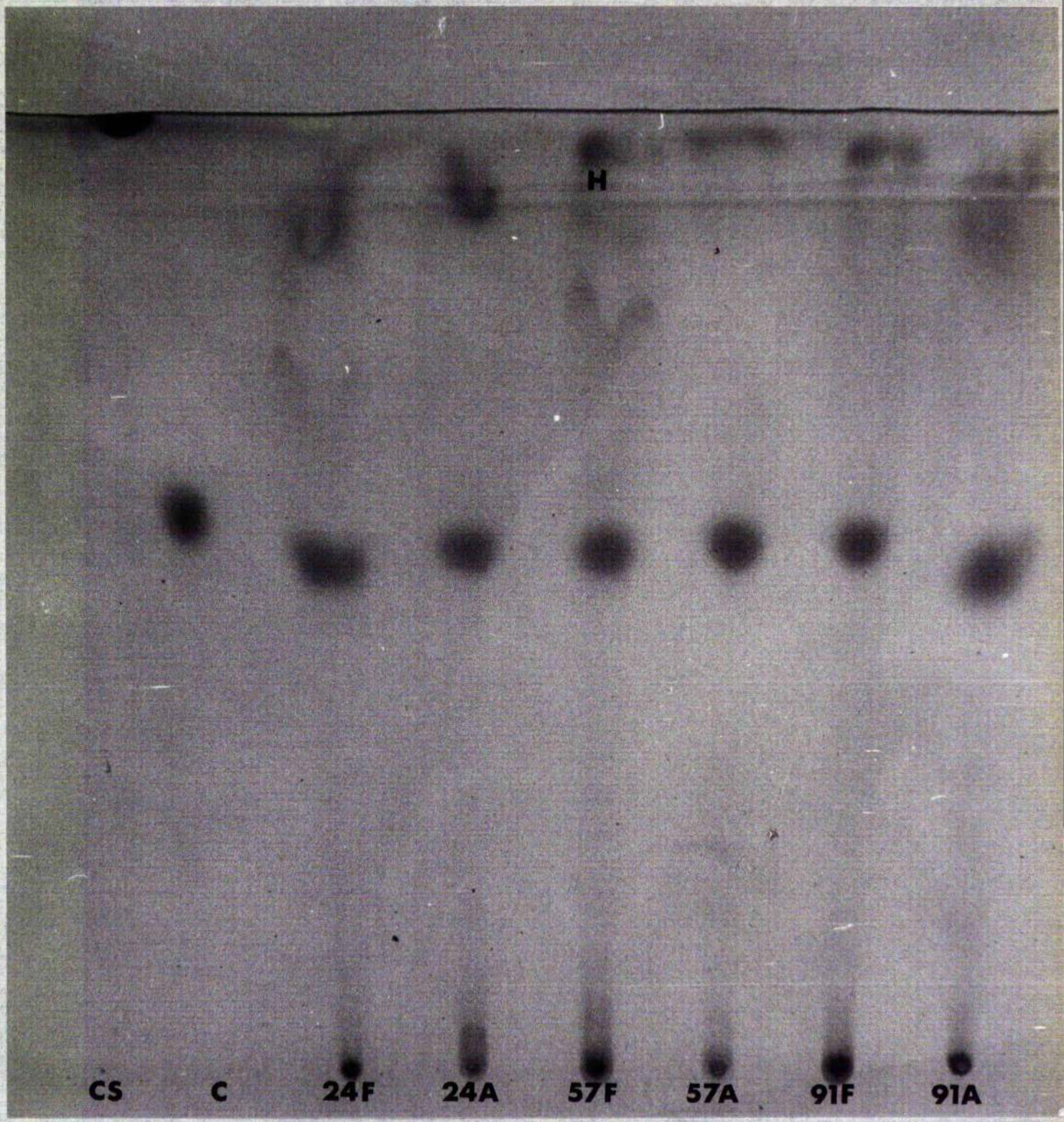
(ii) Recoveries do not include fatty acids constituting less than 1% of the total.

(iii) x:y denotes fatty acid with x carbon atoms and y double bonds.

(iv) Br. denotes branched chain fatty acid.

(v) Several components were unidentifiable by the methods used and where present these have been indicated by their apparent carbon numbers, as determined by G.L.C. on the DEGS column, in parentheses.

Table R.23 - Variation in fatty acid composition of lipid fractions with age.



Key C = cholesterol: CS = cholesterol stearate; H = hydrocarbon material; F = free lipid fraction; A = acid hydrolysed lipid fraction.

Fig. R.11. - Thin-layer chromatogram of non-saponifiable lipid fractions from S. flava membranes.

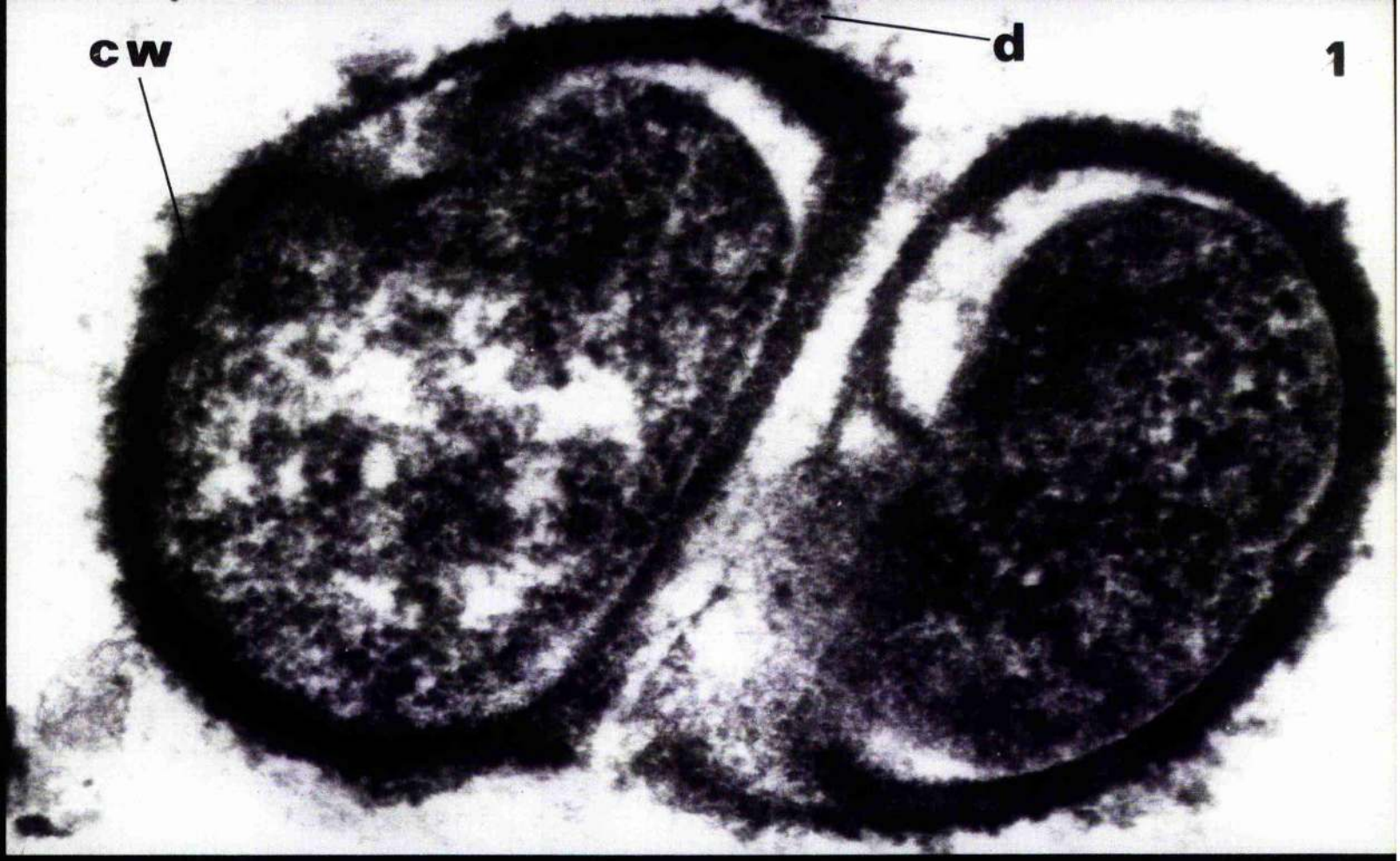
F. ELECTRON MICROGRAPHS OF S. FLAVA AND S. MORRHUAE.

Key to labelling:-

n,	=	Nuclear material	p	=	Polymetaphosphate granule
m,	=	Mesosome	v,	=	Membrane-bound vesicle
cw,	=	Cell wall	dms	=	Double membrane septum
cm,	=	Cytoplasmic membrane	g,	=	Unidentified granular inclusions
r,	=	Ribosomes	s,	=	developing membrane septum.
d,	=	Cell wall debris			

PLATE 1. *S. flava* (lysozyme-treated). Fixation: $KMnO_4$, pH 4. Post-staining: lead citrate/uranyl acetate. X 87,900.

PLATE 2. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 4. Post-staining: lead citrate/uranyl acetate. X 98,000.


cw
d
1

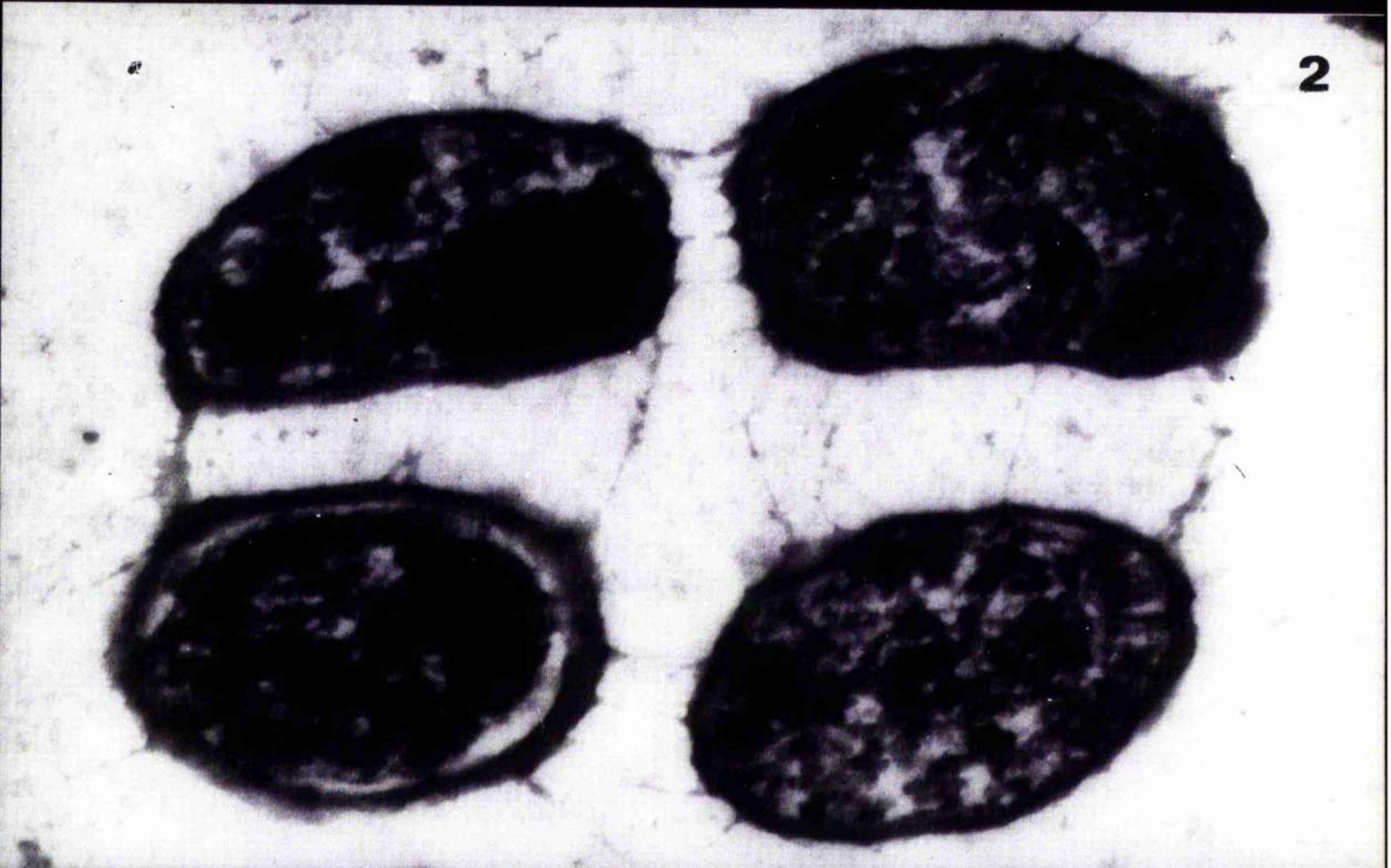
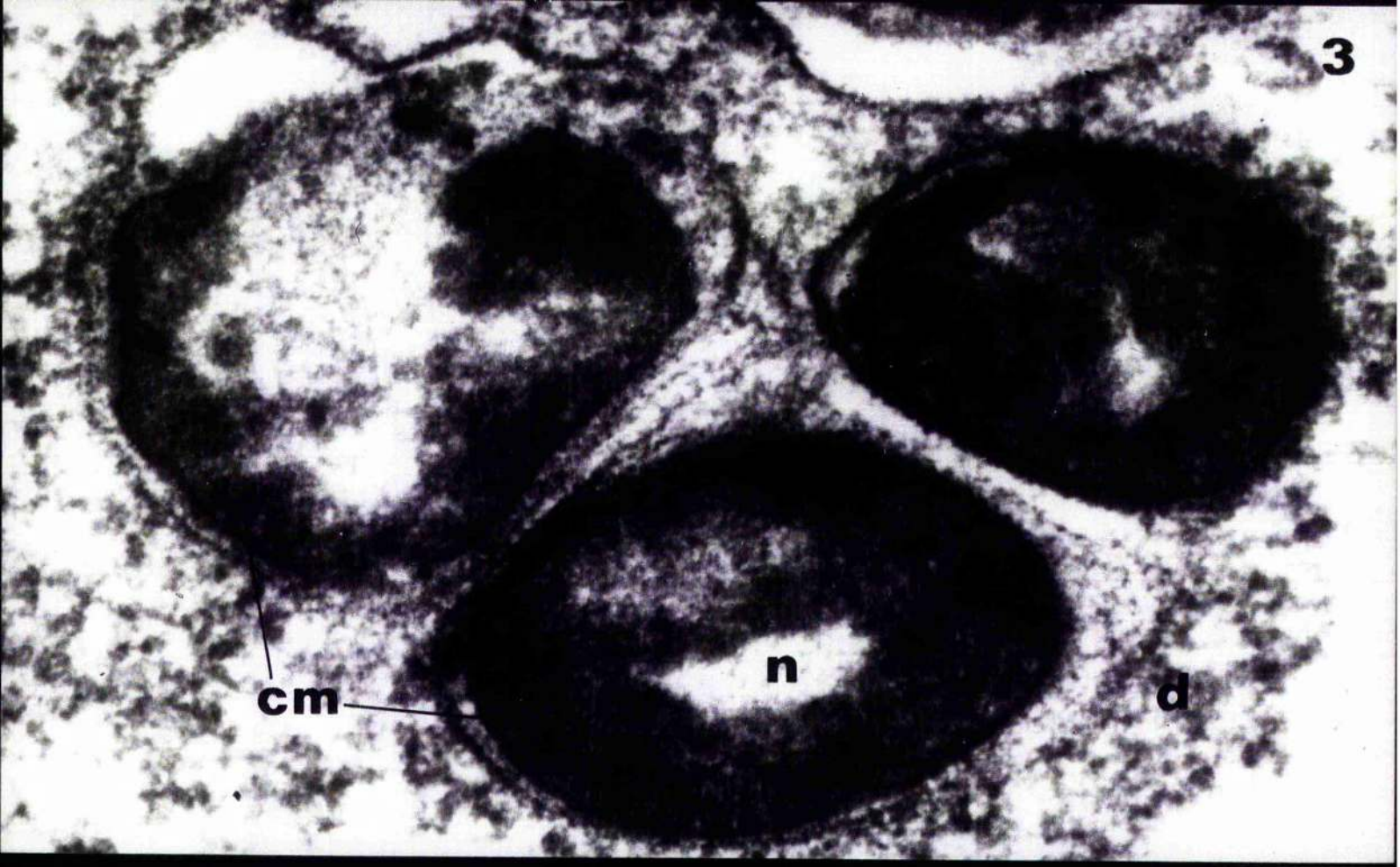

2

PLATE 3. *S. flava* (lysozyme-treated). Fixation: $KMnO_4$, pH 8. Post-staining: lead citrate/uranyl acetate. X 92,000.

PLATE 4. *S. flava* (lysozyme-treated). Fixation: $KMnO_4$, pH 6. Post-staining: lead citrate/uranyl acetate. X 92,000.


3
cm
n
d

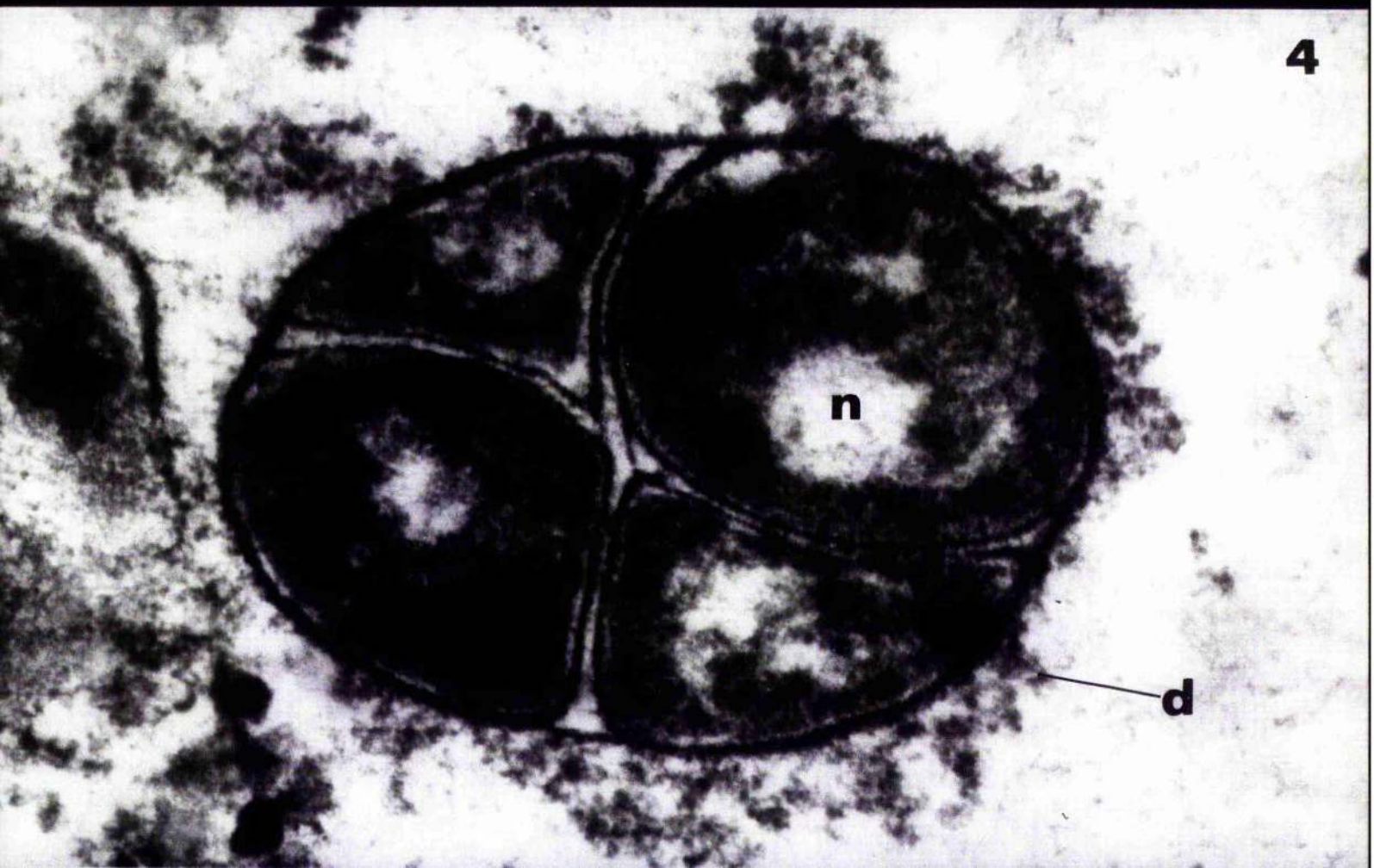
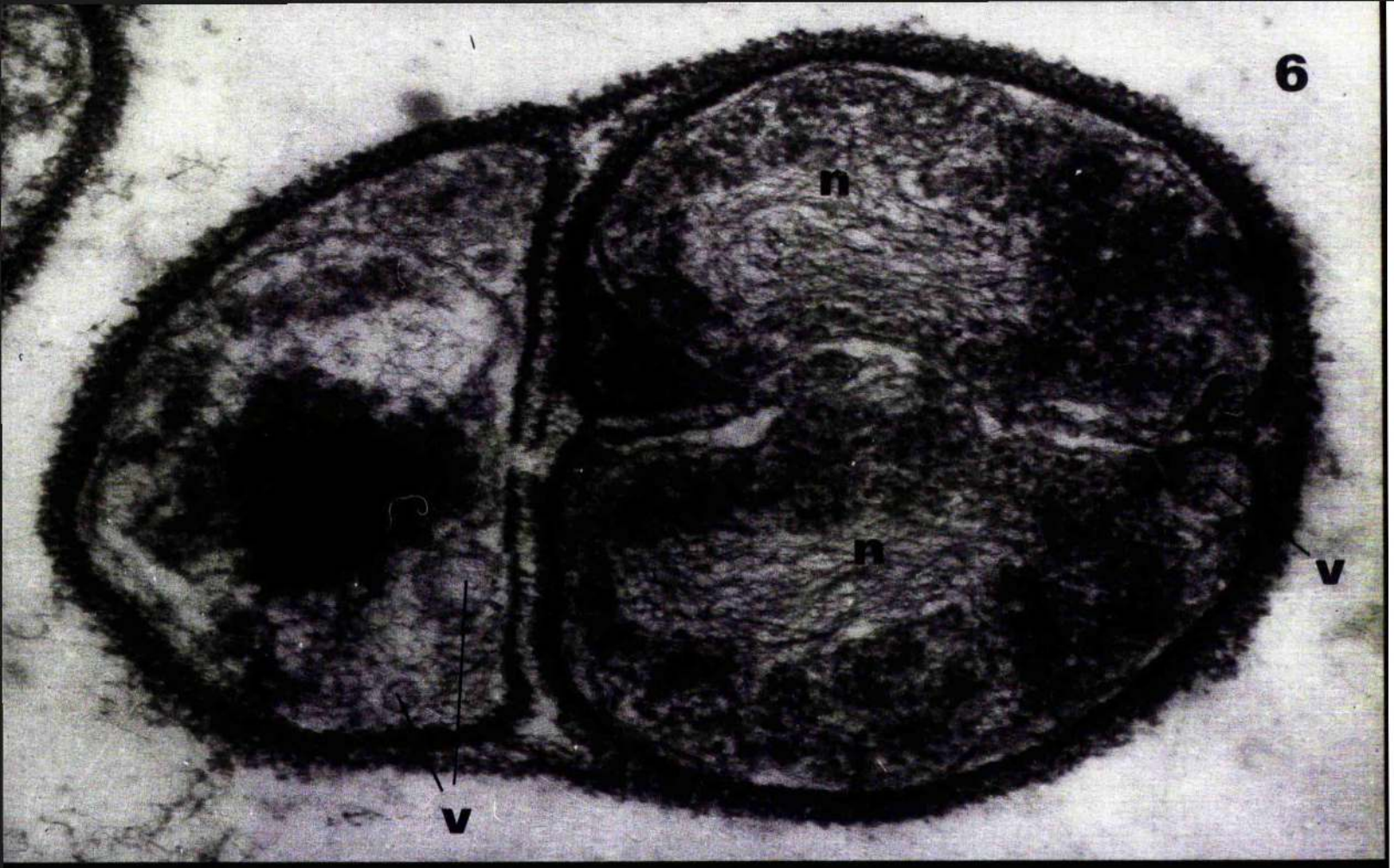

4
n
d

PLATE 5. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 126,000.


cm
5
cw
v
n
n

PLATE 6. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 122,000.

PLATE 7. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 111,000.


6
n
n
v
v


7
n
cw
cm

PLATE 8. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 140,000.


8
n
n
cw

PLATE 9. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 122,000.

PLATE 10. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 77,500.


9
v
cm
v

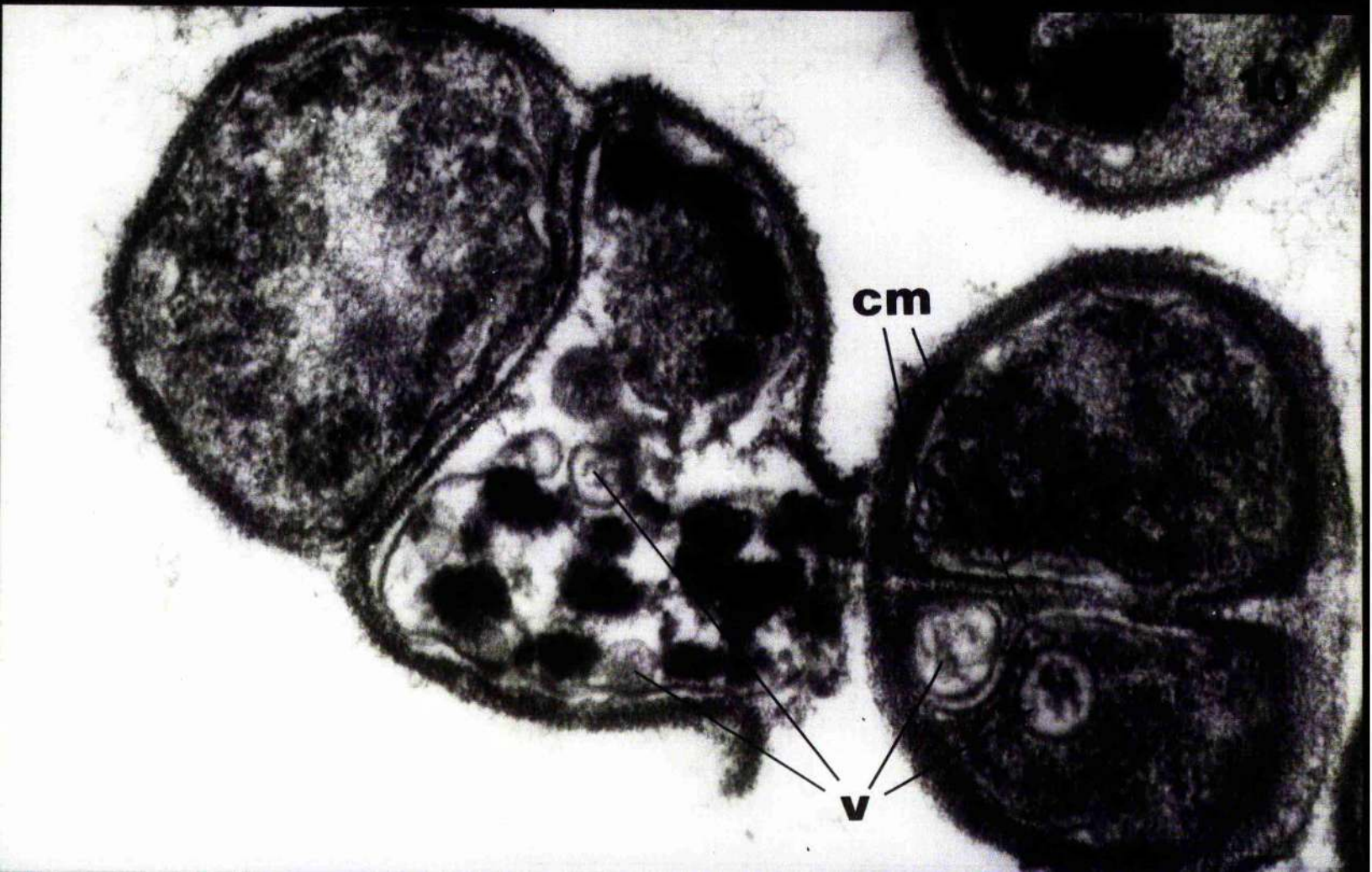

10
cm
v

PLATE 11. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 153,000.


11
cm
v

PLATE 12. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 157,000.


12
v
m

PLATE 13. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 87,200.

PLATE 14. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 77,000.


13
n
cw
v

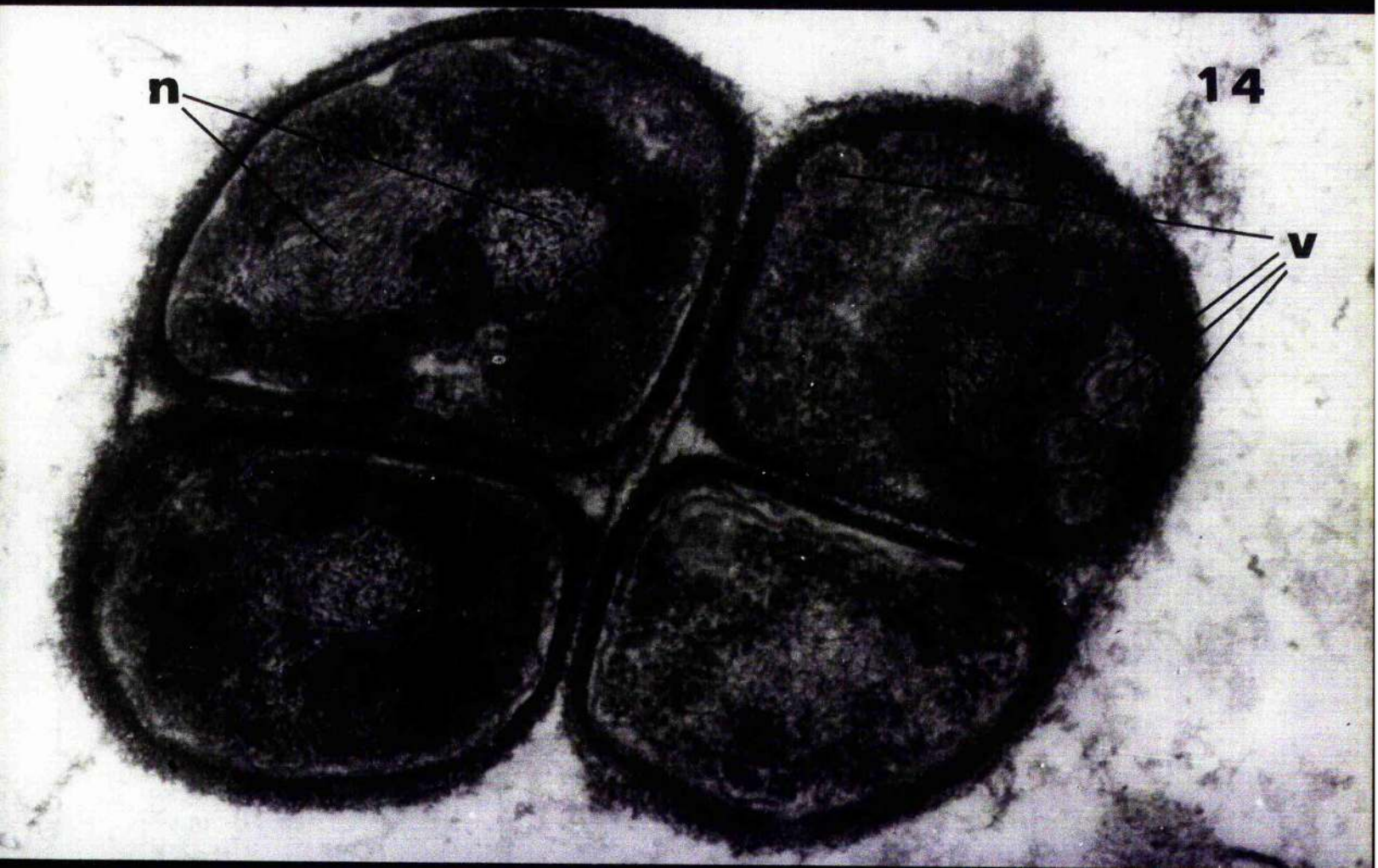
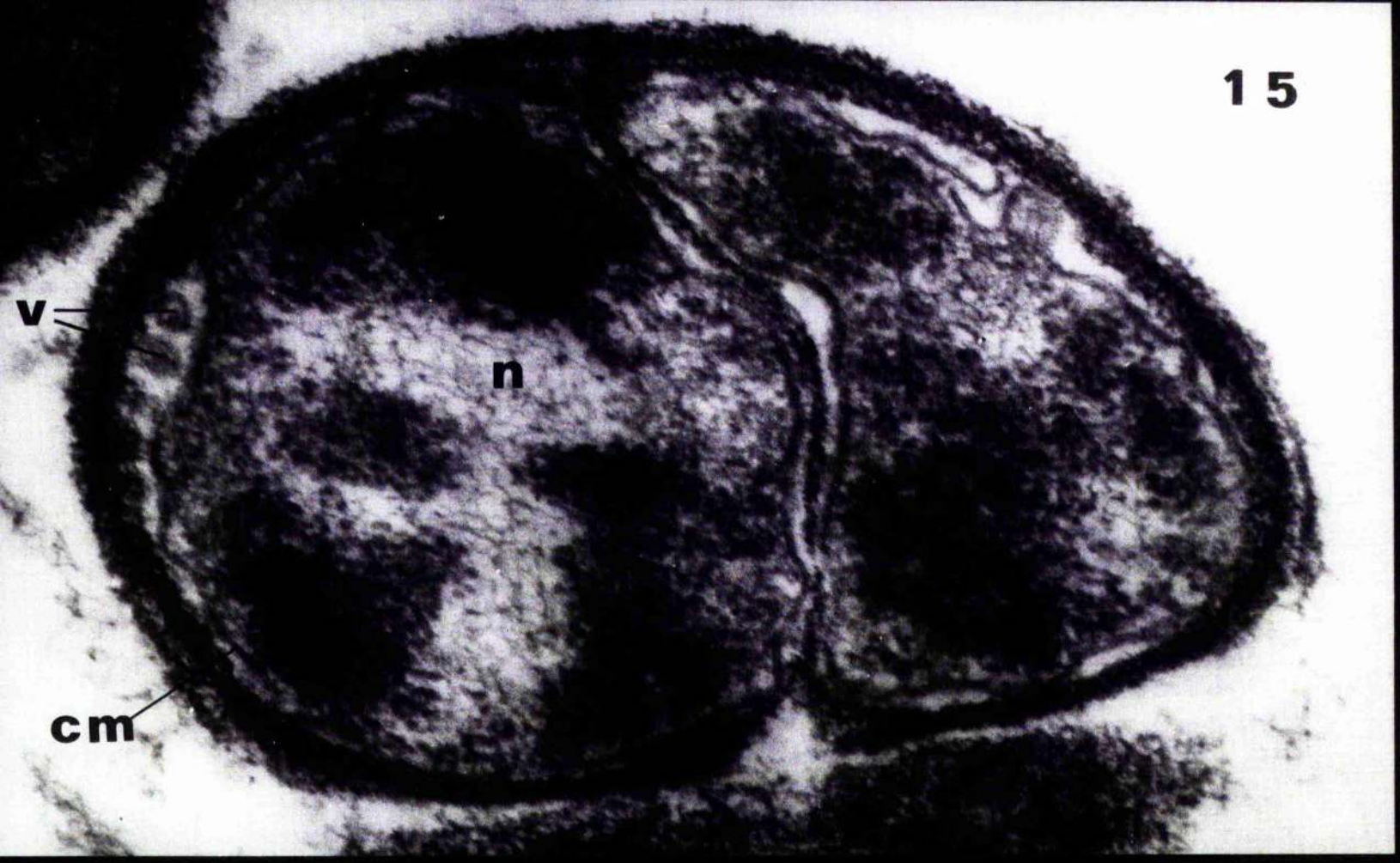

n
14
v

PLATE 15. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 120,000.

PLATE 16. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 105,000.


15
v
n
cm

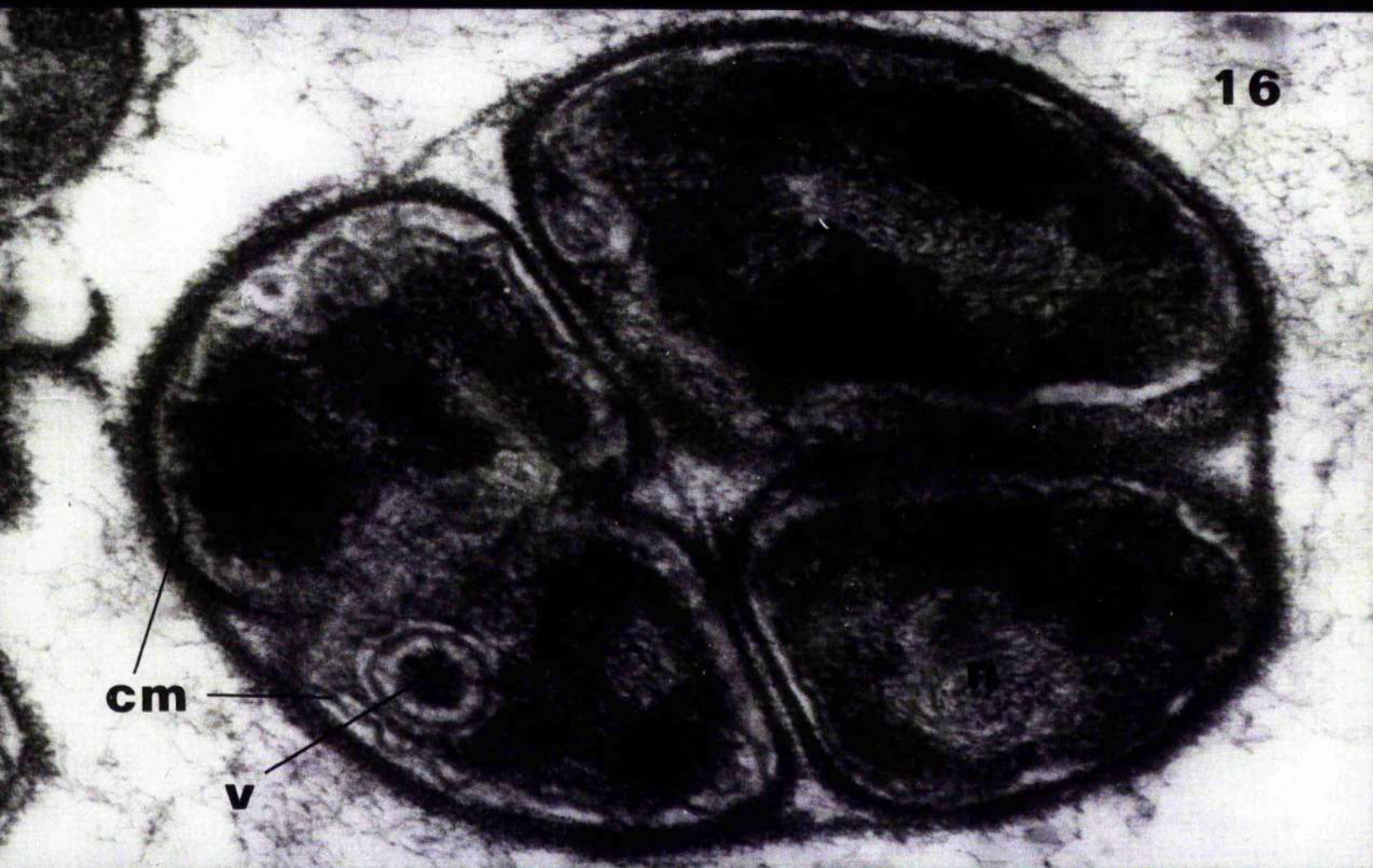

16
cm
v
n

PLATE 17. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 158,500.


cm
17
s
v
n
cw

PLATE 18. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 153,000.


18
cw
n
cw

PLATE 19. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 157,000.

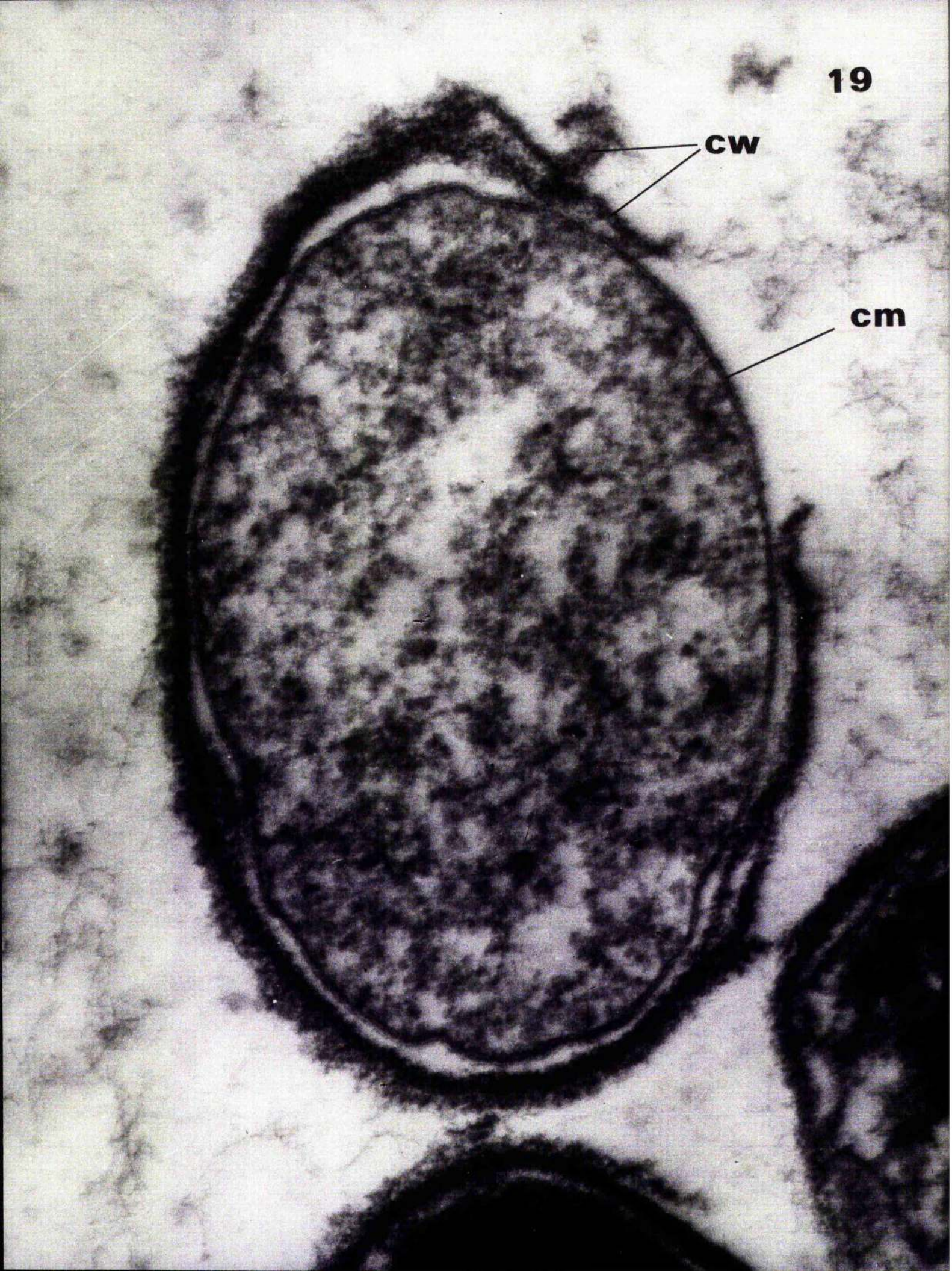
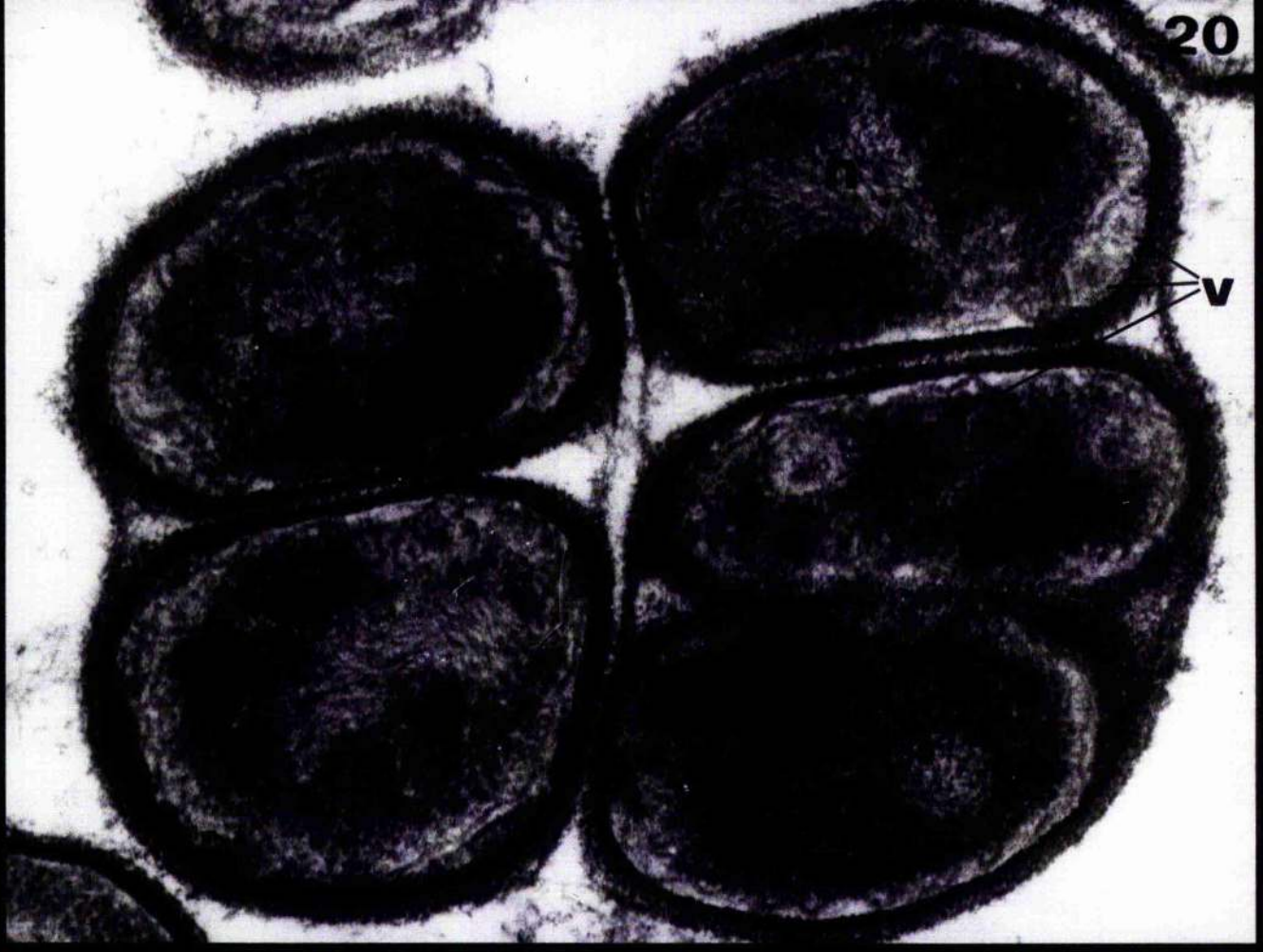

19
cw
cm

PLATE 20. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 83,000.

PLATE 21. *S. flava* (lysozyme-treated). Fixation: OsO_4, pH 8. Post-staining: lead citrate/uranyl acetate. X 129,000.


20
v


21

PLATE 22. *S. flava* spore (?)
Fixation: $KMnO_4$, pH 7.6. Post-staining: lead citrate/uranyl acetate. X 110,000.

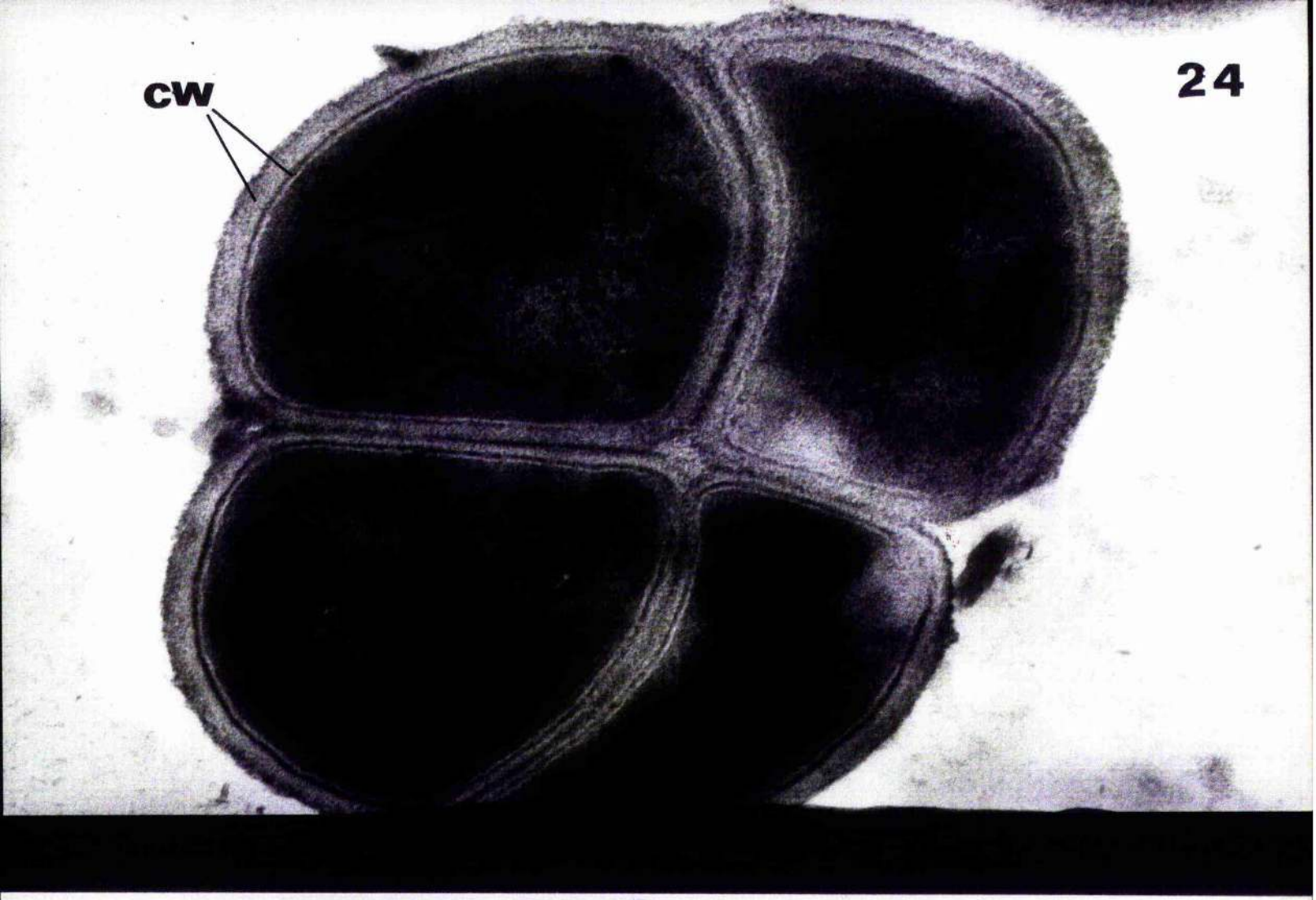
PLATE 23. *S. flava* spore (?).
Fixation: $KMnO_4$, pH 7.6. Post-staining: lead citrate/uranyl acetate. X 82,500.


22
cm
CW
r


23
r
CW
cm

PLATE 24. *S. flava* (whole cells).
Fixation: $KMnO_4$, pH 7.6. Post-staining:
lead citrate/uranyl acetate. X 66,000.

PLATE 25. *S. flava* (whole cells).
Fixation: OsO_4 method I., pH 9.
Post-staining: uranyl acetate.
X 99,000.


cw
24

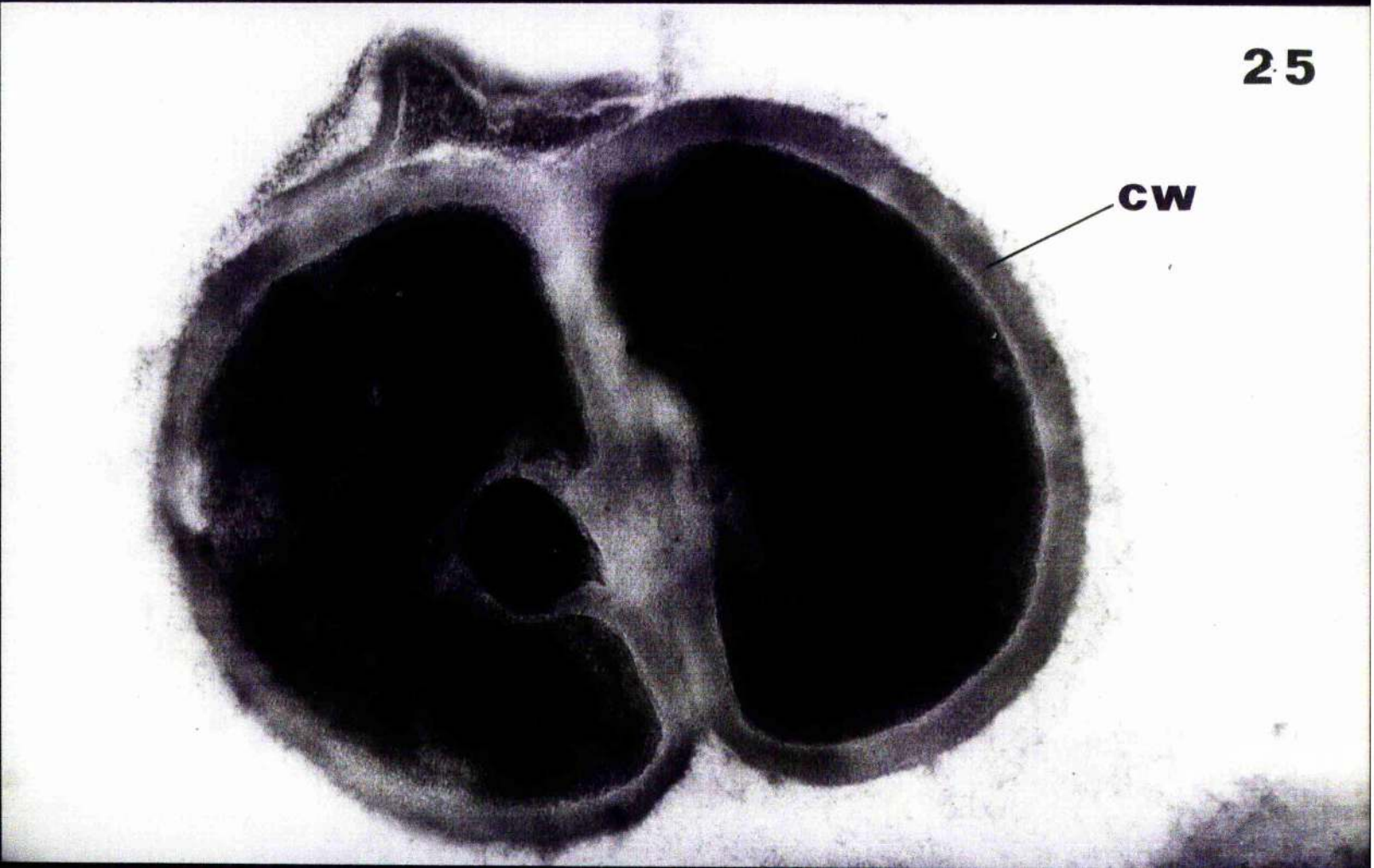

25
cw

PLATE 26. *S. flava* (whole cells).
Fixation: OsO_4 method II., pH 6.0.
Post-staining: lead citrate/uranyl acetate. X 78,500.

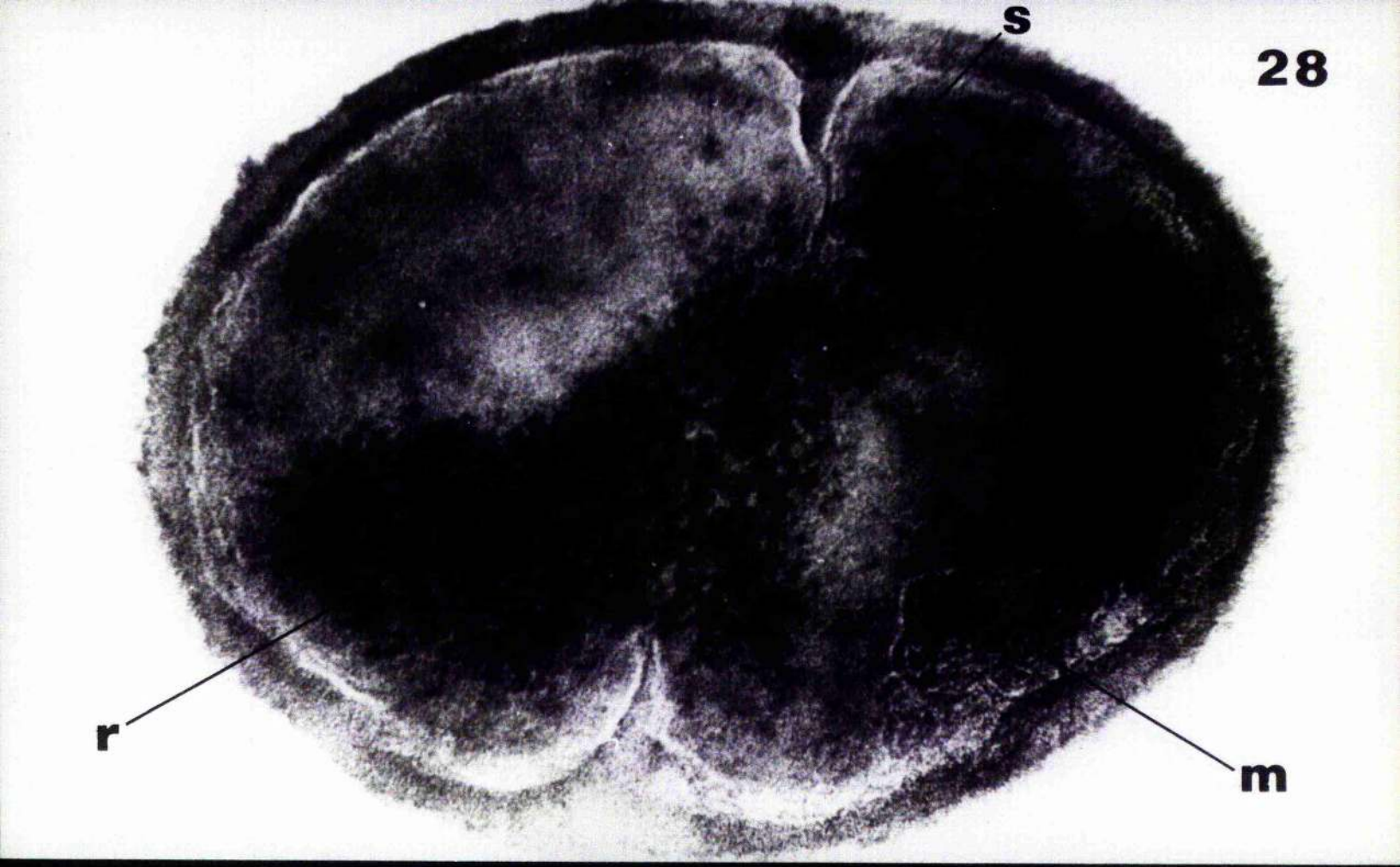
PLATE 27. *S. flava* (lysed cell).
Fixation: OsO_4 method II., pH 9.
Post-staining: lead citrate/uranyl acetate. X 99,000.


26


27
V
S

PLATE 28. *S. flava* (whole cells).
Fixation: OsO_4 method I., pH 9.
Post-staining: uranyl acetate.
X 150,000.

PLATE 29. *S. flava* (whole cells).
Fixation: $KMnO_4$, pH 7.6. Post-staining: lead citrate/uranyl acetate. X 92,000.


s
28
r
m

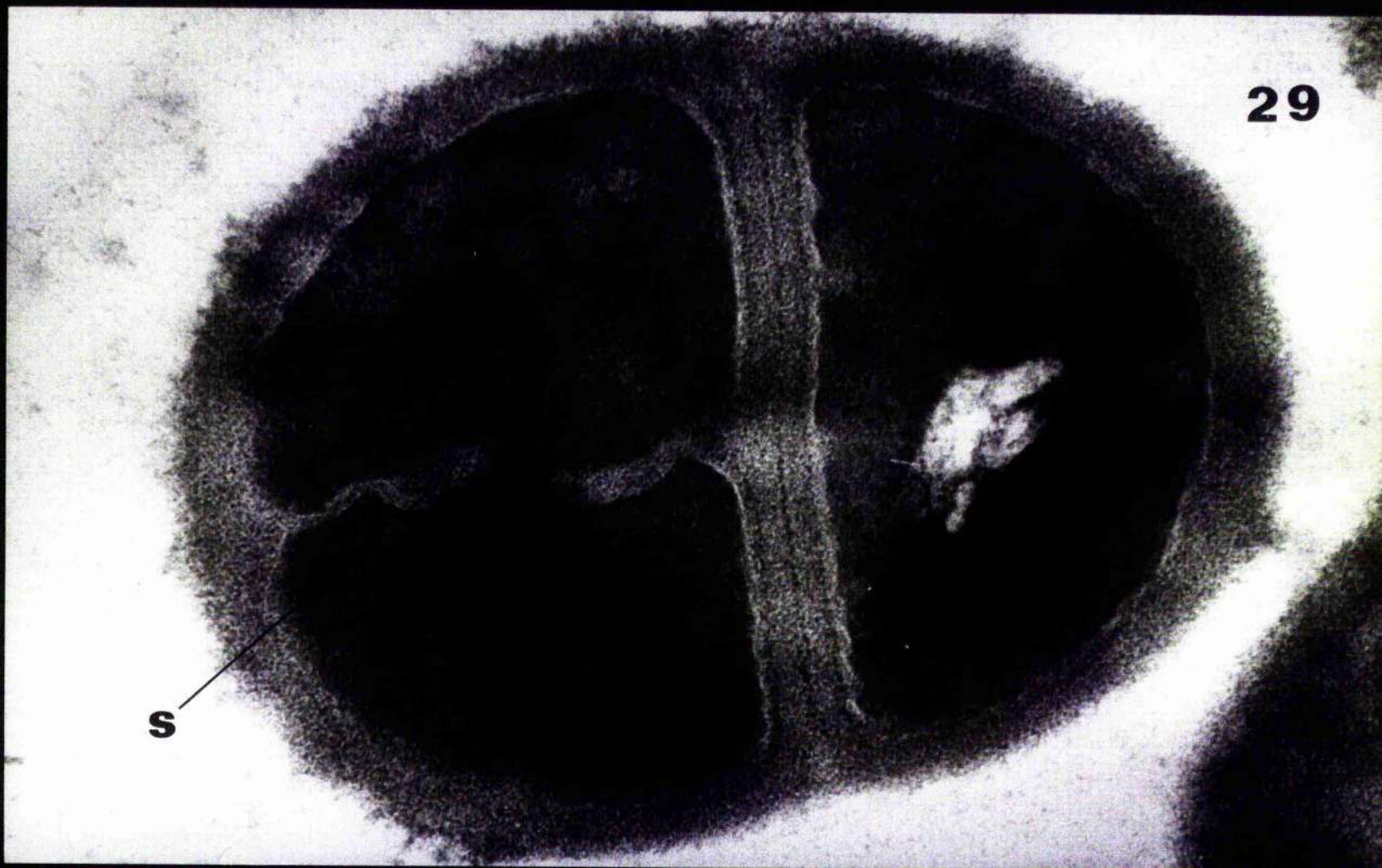
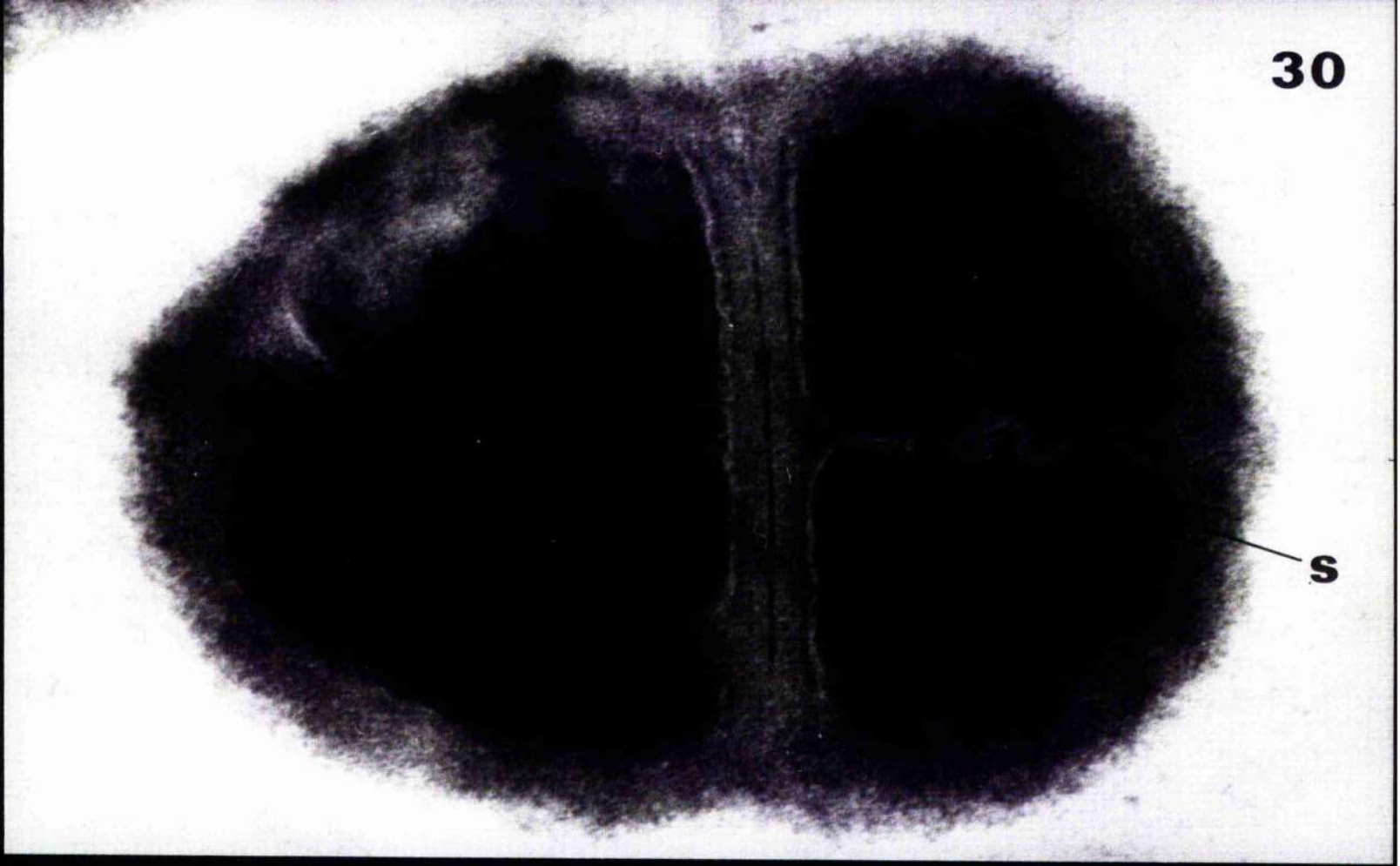

29
s

PLATE 30. *S. flava* (whole cells). Fixation: $KMnO_4$, pH 7.6. Post-staining: lead citrate/uranyl acetate. X 92,000.

PLATE 31. *S. flava* (whole cells). Fixation: OsO_4 method I., pH 9. Post-staining: uranyl acetate. X 161,000.


30
s

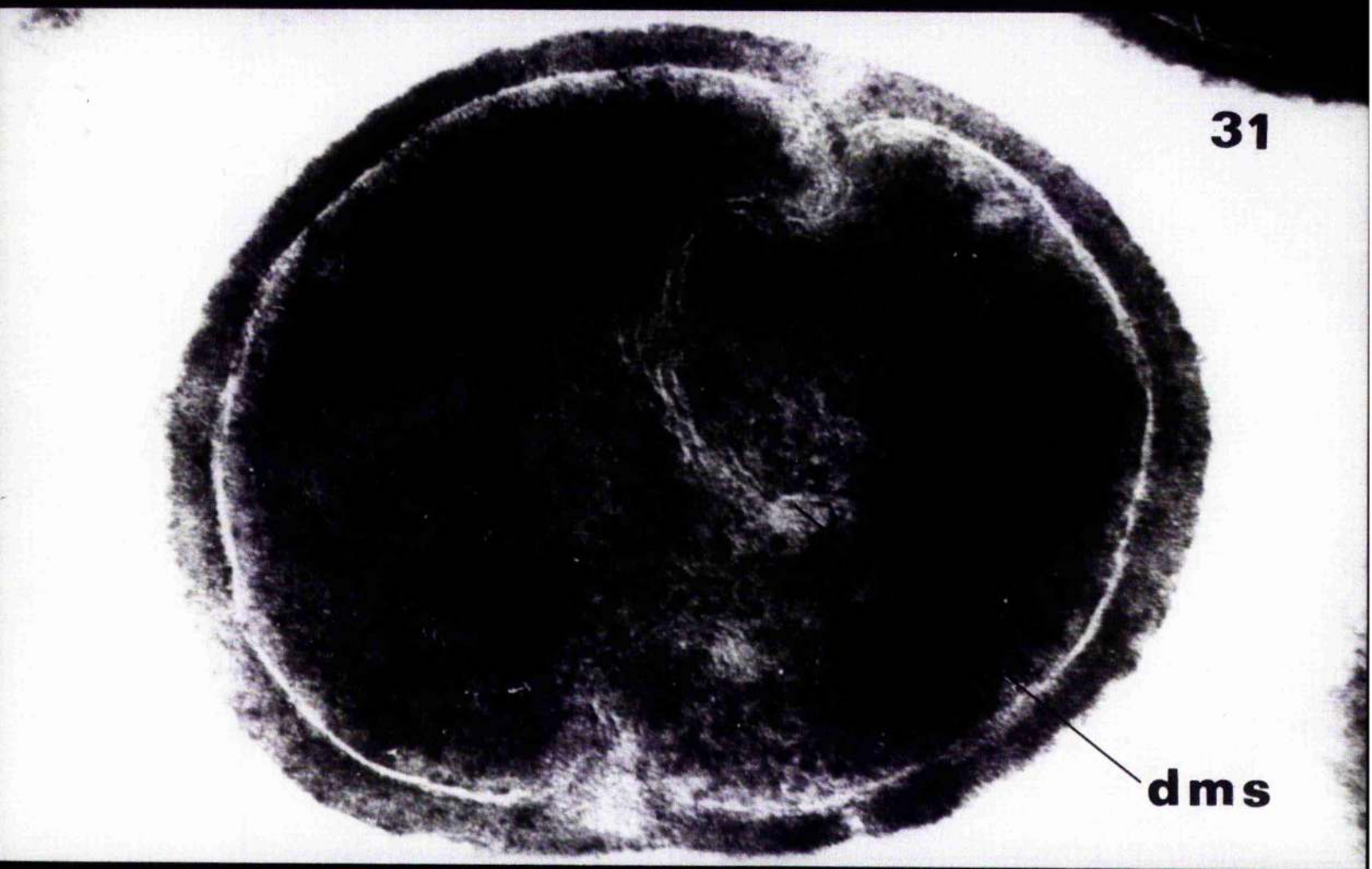
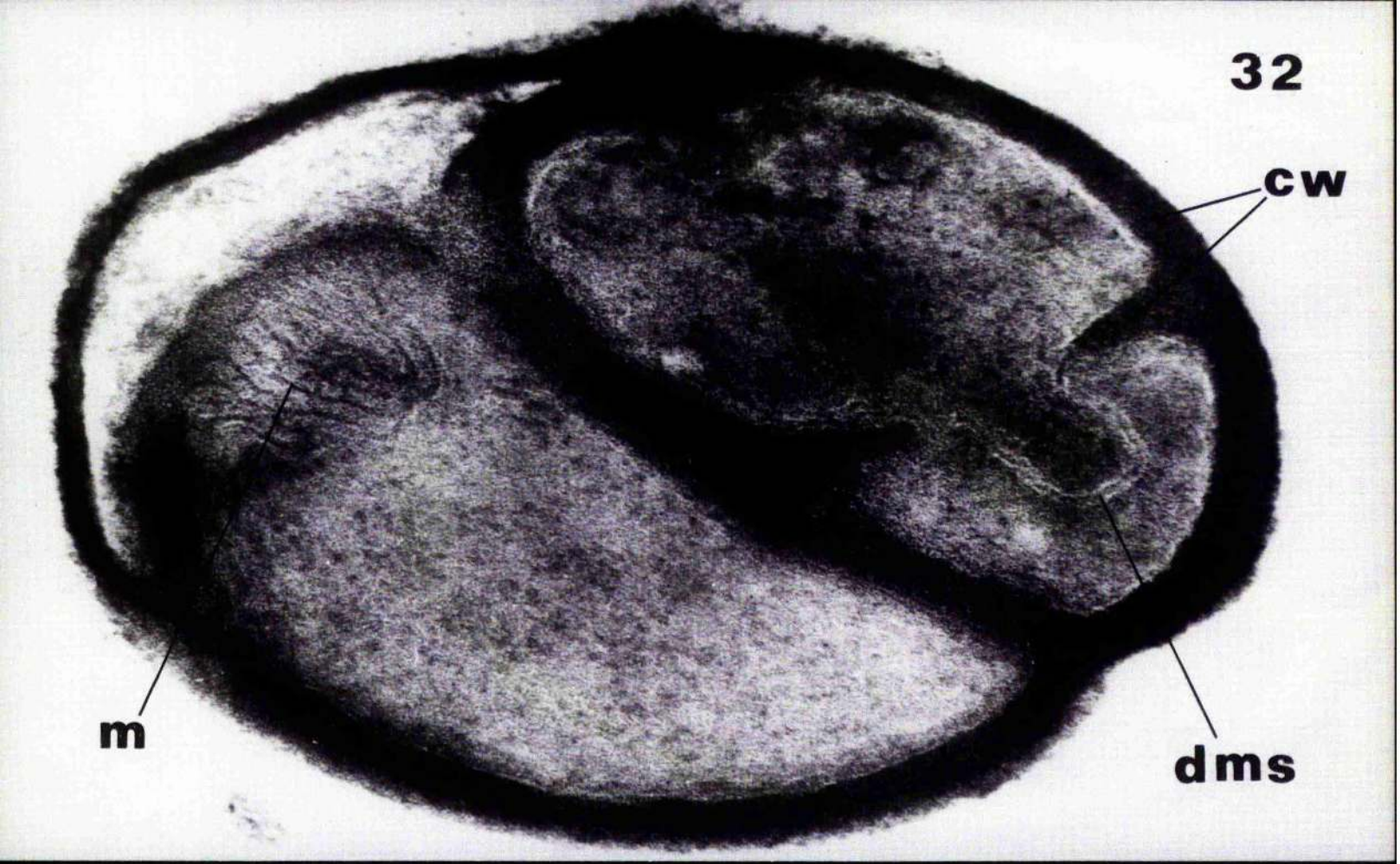

31
dms

PLATE 32. *S. flava* (whole cells).
Fixation: OsO_4 method I., pH 9.
Post-staining: uranyl acetate.
X 128,000.

PLATE 33. *S. flava* (whole cells).
Fixation: OsO_4 method I., pH 9.
Post-staining: uranyl acetate.
X 114,500.


32
cw
m
dms

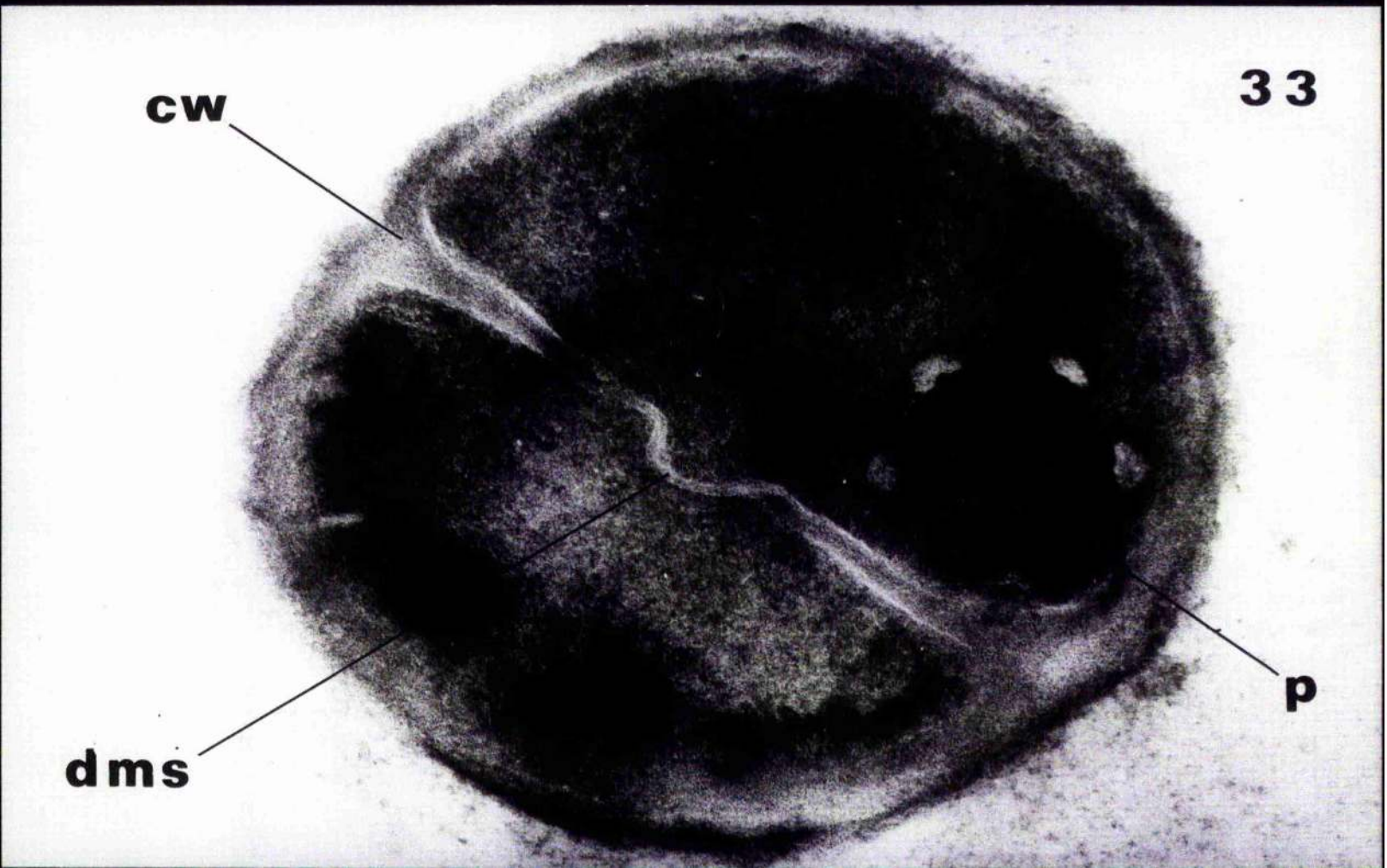

33
cw
dms
p

PLATE 34. *S. flava* (whole cells).
Fixation: OsO_4 method I., pH 9.
Post-staining: uranyl acetate.
X 101,000.

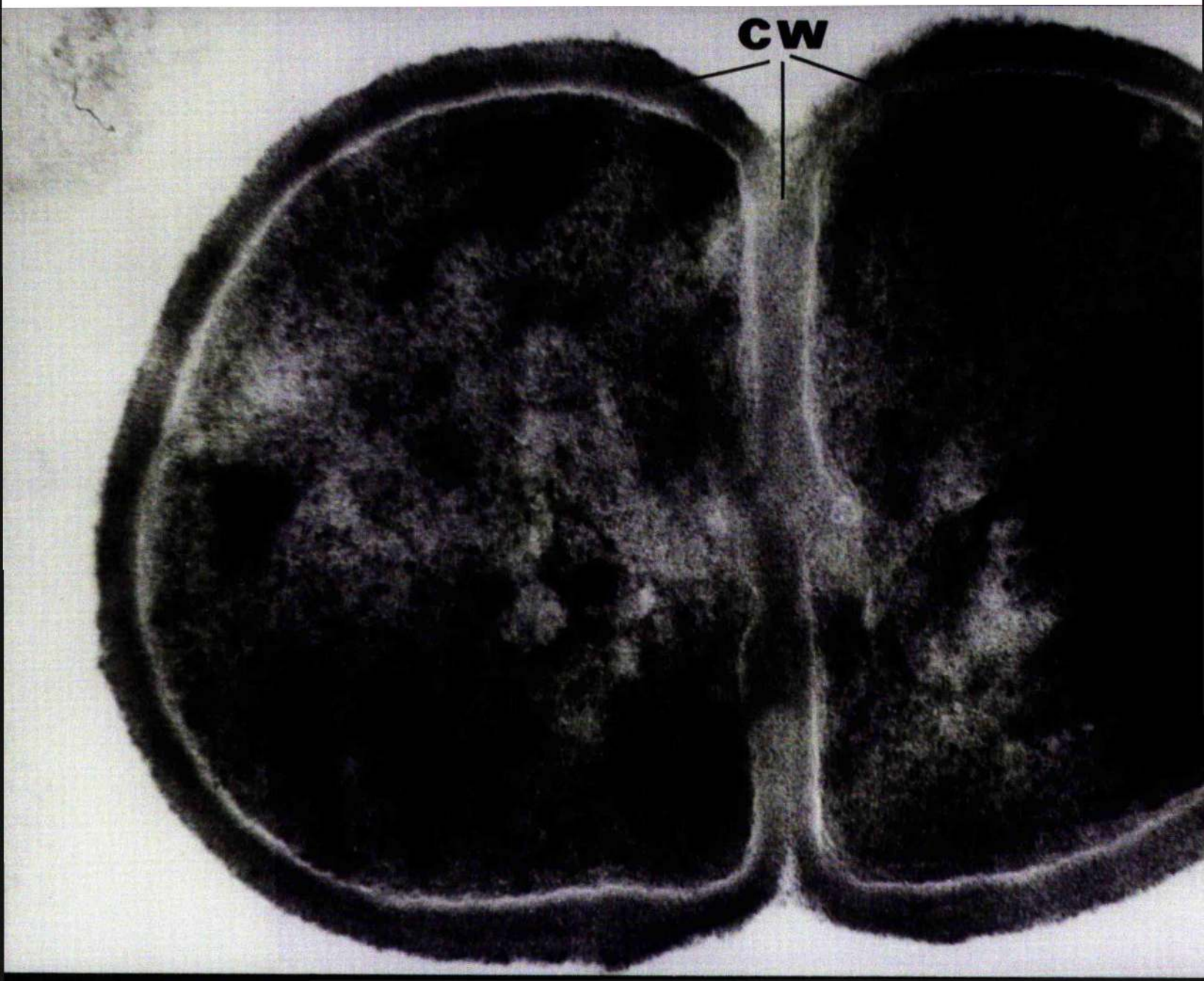

CW

PLATE 35. S. morrhuae. Fixation: OsO_4 method II., pH 6. Post-staining: lead citrate/uranyl acetate. X 33,600.

PLATE 36. S. morrhuae. Fixation: OsO_4 method II., pH 6. Post-staining: lead citrate/uranyl acetate. X 56,500.


35
n


36
n
v

PLATE 37. S. morrhuae. Fixation: OsO_4 method II., pH 6. Post-staining: lead citrate/uranyl acetate. X 85,300.


cw
n
cw

PLATE 38. *S. morrhuae.* Fixation: glutaraldehyde/OsO_4, pH 6. Post-staining: lead citrate/uranyl acetate. X 75,000.

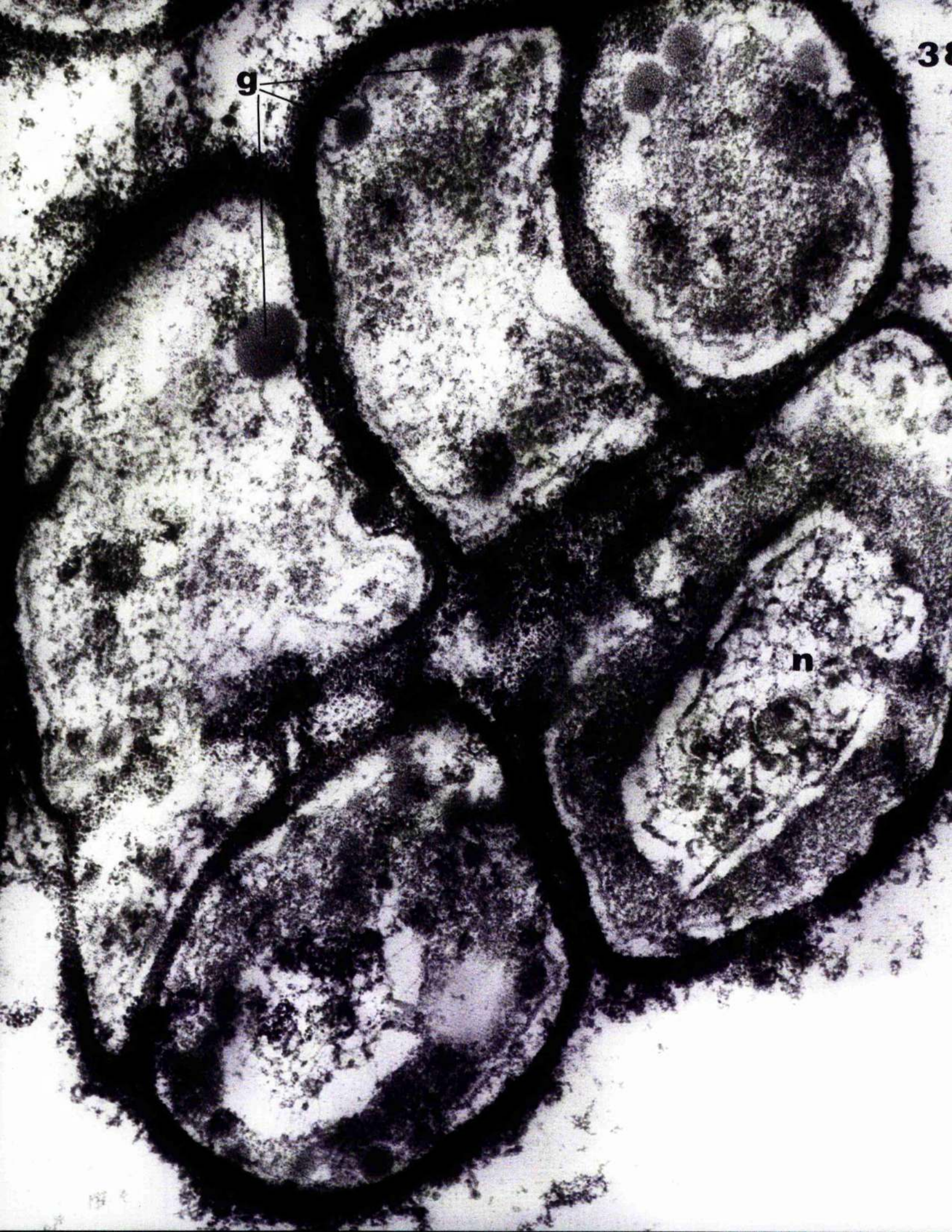

g
n

PLATE 39. S. morrhuae. Fixation: glutaraldehyde/OsO_4, pH 6. Post-staining: lead citrate/uranyl acetate. X 75,000.

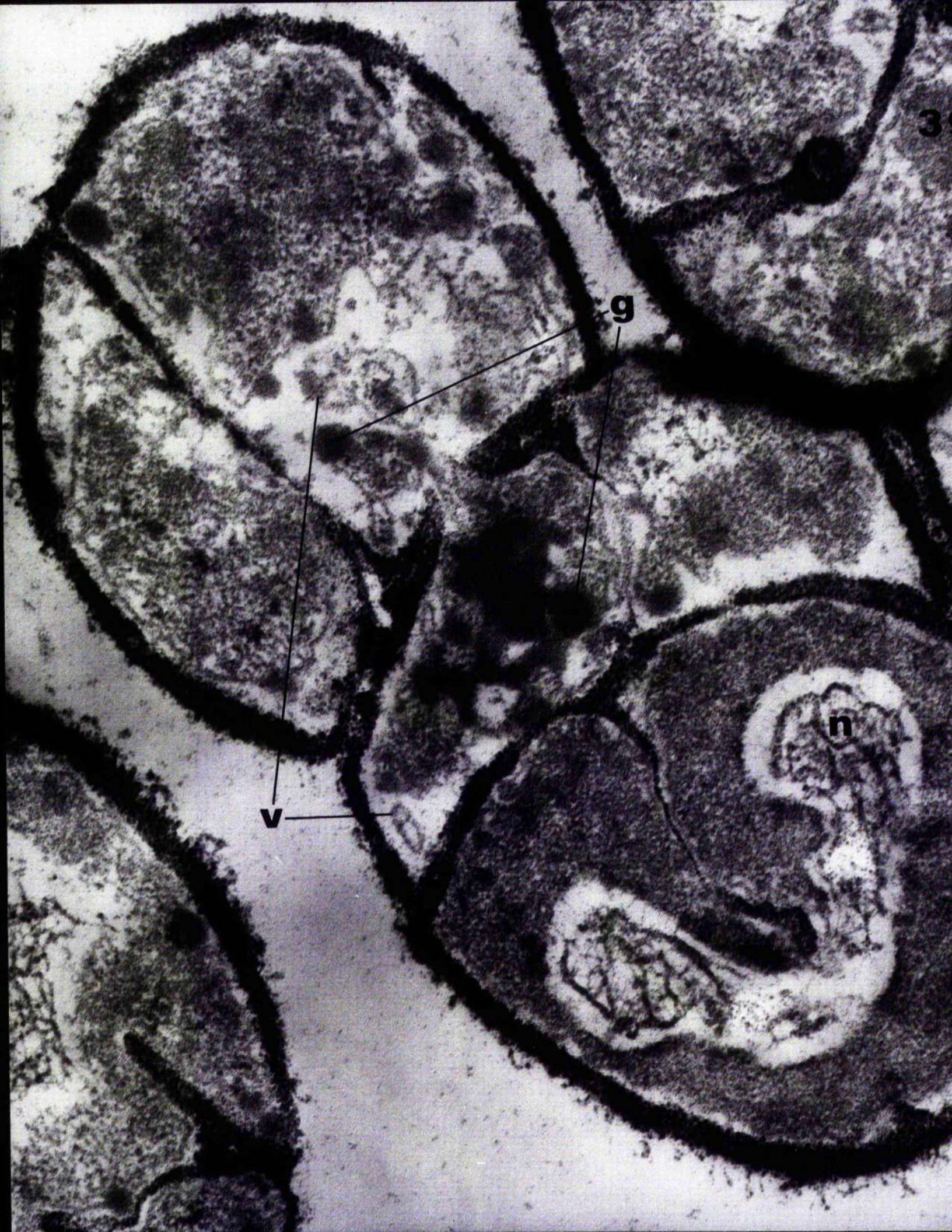

g
n
v

PLATE 40. *S. morrhuae.* Fixation: glutaraldehyde/OsO_4, pH 6. Post-staining: lead citrate/uranyl acetate. X 100,000.

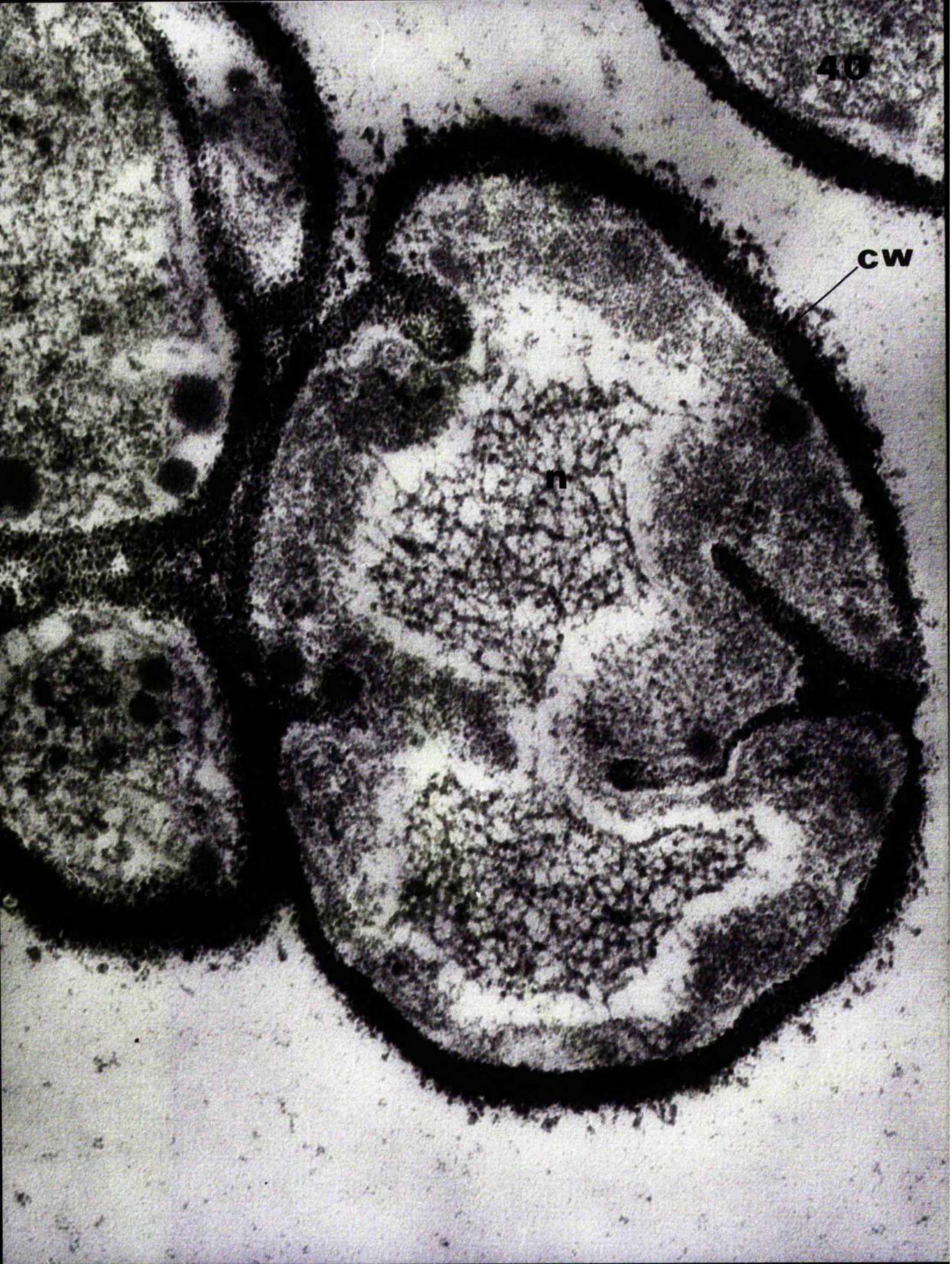
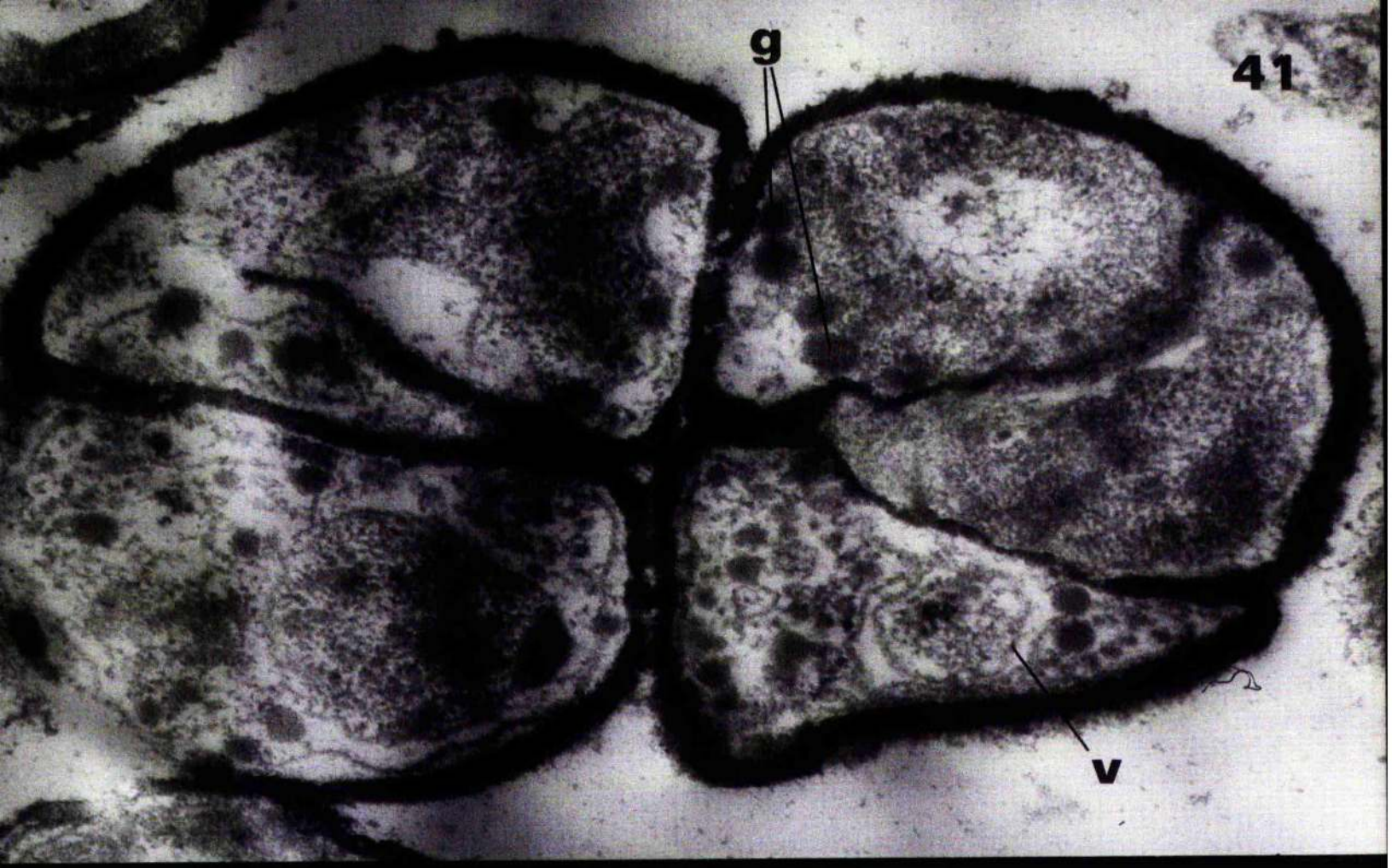

40
cw
n

PLATE 41. *S. morrhuae.* Fixation: OsO_4 method II., pH 6. Post-staining: lead citrate/uranyl acetate. X 56,500.

PLATE 42. *S. morrhuae.* Fixation: OsO_4 method II., pH 6. Post-staining: lead citrate/uranyl acetate. X 56,500.


g
41
v

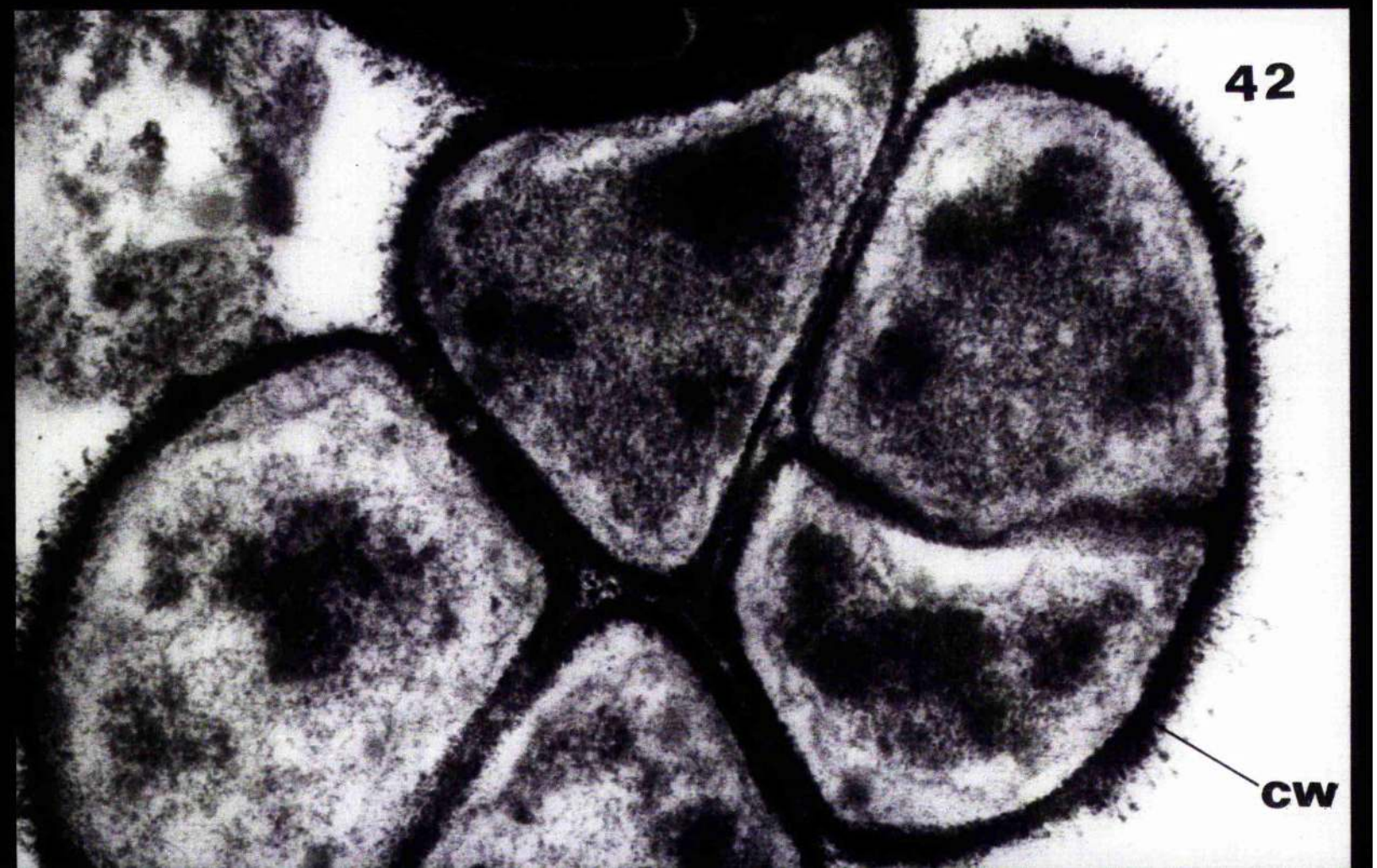

42
cw

PLATE 43. S. morrhuae. Fixation: OsO_4 method II., pH 6. Post-staining: lead citrate/uranyl acetate. X 56,500.

PLATE 44. S. morrhuae. Fixation: OsO_4 method II., pH 6. Post-staining: lead citrate/uranyl acetate. X 91,000.


cm
43
g


cm
44

DISCUSSION

A. CAROTENOID GLYCOPEPTIDES/GLYCOPROTEINS FROM S. FLAVA AND S. MORRHUAE

The detergent-solubilised fraction from S. flava membrane was not resolved into subfractions by any of the methods employed and the single symmetrical peak observed on the analytical ultracentrifuge confirms the findings of several others as to the ultracentrifugal homogeneity of detergent-solubilised membrane components (Salton, 1965c; Razin, 1967; Smith et al., 1969). Salton (1967) also found that detergent-solubilised membrane fractions from S. lutea and M. lysodeikticus were not resolvable by either sucrose gradient electrophoresis or TLC on G100 Sephadex. However, Smith et al. (1969) were able to detect at least 11 bands after disc-gel electrophoresis of detergent solubilised material from M. laidlawii. The material isolated from S. flava contained only traces of any basic amino acid residues, and the high ammonia peak obtained on amino acid analysis suggests that aspartic and glutamic acids were present predominantly as their amides. These findings provide a plausible explanation for the non-migration of the material under either of the electrophoretic techniques used, since the net charge on the peptide moiety would be negligible. This would also explain why no satisfactory titration curve could be obtained for the material.

The choice of Lubrol L as the detergent to be used in this work was made after a careful quantitative study of the solubilising efficiency of some 20 anionic, cationic and non-ionic detergents, with respect to S. flava membranes. It was apparent

from this that the non-ionic preparations, and especially Lubrol L, were far more efficient than either type of ionic detergent, but that even Lubrol L did not produce better than 70% solubilisation of the membrane. Salton (1965c) found that the non-ionic detergent Nonidet P40 was more efficient than the ionic detergent sodium dodecyl sulphate (SDS), and was able to achieve a greater than 95% solubilisation of membranes from S. lutea and M. lysodeikticus using Nonidet P40. Since Nonidet P40 and Lubrol L are of similar chemical nature, being polymeric condensation products of ethylene oxide and nonyl phenol, it may be inferred that the composition of the membranes from S. flava must differ from that of the membranes from S. lutea and M. lysodeikticus to account for the significant differences in the degree of solubilisation obtained. Bishop et al. (1967) have already observed that the chemical composition of bacterial membranes is of considerable influence on the solubilising ability of detergen and both these workers and Razin et al. (1965) have achieved 100% solubilisation of membranes using SDS. It is interesting to not that, whereas it has been shown in this work for S. flava and by Salton et al. (1965c) for S. lutea and M. lysodeikticus, that better solubilisation of the bacterial membrane is obtained with non-ionic detergents, for membranes from other species, e.g. B. subtilis (Bishop et al., 1967a) and M. laidlawii (Razin et al., 1965), ionic detergents exhibit a greater efficiency. This presumably is again a reflection of differences in membrane composition between the species. The component of the membrane which

is chiefly responsible for binding with the detergent and which hence dictates the efficiency with which the membrane will be solubilised, appears also to vary. Bishop *et al.* (1967a) showed that treatment of *B. subtilis* membranes with SDS selectively solubilised protein, leaving a lipid-enriched residue whereas treatment of *S. lutea* membranes with deoxycholate (Salton, Freer & Ellar, 1968), and treatment of *M. laidlawii* membranes with SDS (Razin *et al.*, 1965) is said to selectively extract lipid, leaving a residue with a much increased protein/lipid ratio, as compared with the original membrane.

The material isolated from *S. flava* is highly stable to heat and extremes of pH value (4 - 10) and although the association of carotenoid and protein will undoubtedly confer mutual stability on these components (c.f. Cheesman *et al.*, 1967), in view of the high detergent content of the material, the inclusion of these membrane components within detergent micelles may also contribute to this stability. Although exhaustive dialysis was employed as a step in its preparation, the purified material was found to still be rich in detergent and it proved impossible to remove all traces of Lubrol by either partition between a variety of immiscible solvent systems, or selective precipitation with e.g. acetone, $(NH_4)_2SO_4$, TCA, since under these conditions the detergent co-precipitated with the membrane material. Others, including Bishop *et al.* (1967a), have also shown that it is impossible to remove all traces of detergent by dialysis, but these workers did show, using ^{35}S labelled SDS that it was

possible to achieve detergent removal using G25 Sephadex. However removal of Lubrol L, probably by virtue of its non-ionic nature, was not possible by this method. Gaylor and Delviche (1969) have devised a method for the removal of non-ionic detergents from proteins using LH20 Sephadex. This method was not tried in this work but may well be applicable to the separation of Lubrol L from the solubilised membrane fraction from S. flava.

Release of free pigment from the Lubrol L solubilised material proved extremely difficult and would seem to indicate a stable bond which is almost certainly covalent, and may be glycosidic in view of its slight alkaline lability, between the carotenoid and the remainder of the material. At the same time, the possible protective influence of the inclusion of the materia within detergent micelles on the bonding with carotenoid must be considered. Papain digestion rendered much of the pigmented material ether soluble, although this digested material still contained detergent, glucose and a short peptide. Since the effect of papain digestion would be to hydrolyse some of the peptide bonds in the protein component of the material, the preferential solubility of the digested material in ether is presumably caused by the removal of a large portion of hydrophilic protein which may also be enclosed within a detergent micelle and would hitherto have been responsible for the water solubility of the complex.

The shapes of the plots of the data obtained from

osmometry for the material in the presence and absence of KCl, and the large discrepancy between the $\bar{M}_n$ values indicate that the membrane components adsorb large numbers of detergent micelles and in doing so become so large in size that the colligative properties of the material become effectively those of the detergent itself. The presence of salt induces the aggregation of already existing micelles and/or the formation of new micelles from free detergent present in solution and this produces an anomalously high value for the molecular weight of the material as determined by osmometry. The molecular weight of the material as indicated by the S value is somewhat lower than that determined by osmometry, but since the work of Smith et al. (1969) has shown that the S value of a detergent solubilised membrane preparation from M. laidlawii is dependant on the detergent concentration, and as such is a reflection of the properties of the detergent rather than of the membrane components, the significance of the sedimentation coefficient would seem open to doubt.

Furthermore, the interpretation of single, symmetrical sedimentation patterns produced by other detergent-solubilised membrane fractions (Razin, 1967; Salton, 1967b; Butler et al., 1967), to indicate that identical membrane sub-units had been isolated would also seem dubious, since the sedimentation patterns may merely represent heterogeneous membrane components bound within detergent micelles of the same size.

Other workers (Wolken & Schwertz, 1956) have determined molecular weights in the presence of detergent, but this

practice would not seem advisable, in view of the findings presented here.

Both subfractions of the polar carotenoid fraction from S. flava were shown to contain carotenoid, glucose and amino acids. The carotenoid moiety is assumed to be the most polar free carotenoid isolated by Thirkell et al. (1967), which is a C_{50}-dihydroxy-carotenol and is probably identical with sarcinaxanthin (Jensen, 1970 - see Introduction). From these findings it seems likely that this free carotenoid becomes bound to glucose and peptide, to form an integral part of the structure of the membrane, where all the carotenoid pigment is localised in S. flava (Strang, 1968). Thus the following biosynthetic route would seem to operate in this organism:-

Colourless C_{50} precursors ----> C_{50} carotene ---> C_{50} - monohydroxy-carotenol ---> C_{50}-dihydroxy-carotenol ---> C_{50}-carotenoid-glycopeptide ---> integral membrane component.

At the present time, it is not known how many intermediat steps are involved in the addition of glucose and peptide to the dihydroxy carotenoid, but the reported isolation of a monoglucoside of sarcinaxanthin from S. lutea (Norgard et al., 1970) may mean that in S. flava also a monoglucoside is formed first, followed by the diglucoside and finally the attachment of the amino acids, either singly or as a pre-formed peptide. It is possible that the other two subfractions (ii & iv) of the polar carotenoid may correspond to two such intermediates in this process, but neither has yet been prepared in sufficient quantity

to permit their further characterisation.

The molar ratio of glucose to carotenoid in both sub-fractions (i & iii) is 2:1 and the experimental evidence suggests that each hydroxyl function on the carotenoid molecule is involved in a glycosidic linkage with a single glucose molecule. Each glucose residue is in turn linked through one of the remaining primary or secondary hydroxyls to peptide. If one considers how such a complex molecule might be orientated in the bacterial membrane, then the sugar and amino acid residues will be associated with the hydrophilic regions of the membrane whereas the conjugated hydrocarbon chain of the carotenoid must be aligned somehow in the hydrophobic region of the membrane. One such orientation for carotenoid has already been suggested by Smith (1969) for the micellar membrane of M. laidlawii. If the dihydroxy-carotenol is represented as: O----------O , where O = hydrophilic hydroxyls and ---------- = hydrophobic hydrocarbon chain, then this could be accommodated within the architecture of a bimolecular leaflet type of membrane in the manner shown in Fig. D.1.A. Formation b) can be excluded from consideration since for the carotenoid molecule to adopt such a configuration would entail the central double bond being cis, whereas from the I.R. data, all the double bonds are known to be trans. Formation a) therefore is more feasible, and the dotted lines would represent the type of material of which the sub-fractions are constituted. The calculated length of the carotenoid molecule would be of the right order to straddle the lipid

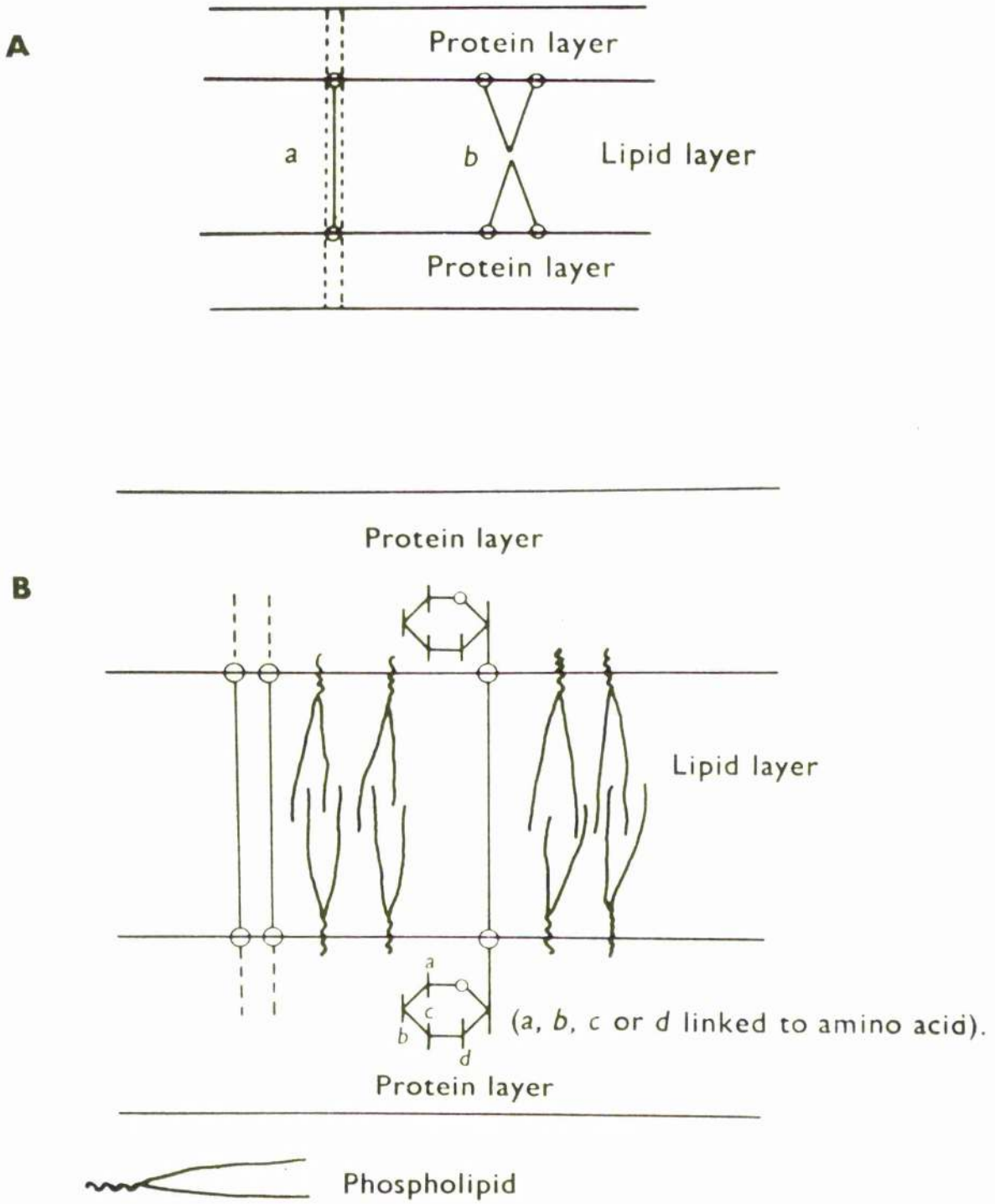


Fig. D.1. - Proposed model for the in vivo orientation of carotenoid in the membrane of S. flava.

layer in this way. Fig. D.1.B. is a more detailed representation of the proposed model for the in vivo state of the carotenoid-glycopeptide material in the membrane. It should be noted that for the model to be tenable, and if the carotenoid moiety is in fact sarcinaxanthin, as has been suggested, then the structure for sarcinaxanthin proposed by Jensen (1970) would have to prove to be the correct one since in that proposed by Arpin et al. (1970), the distance between the hydroxyl functions is not sufficient to bridge the lipid layer of the membrane (see Fig. I.II.).

If the analytical results obtained for the subfractions are considered in relation to the model just proposed, the following observations can be made to support the structure postulated:-

1. The molar ratio of glucose to carotenoid is 2:1.
2. The linkage between carotenoid and glucose is almost certainly glycosidic.
3. Linkage of glucose to peptide is demonstrated by the detection of glucopeptides in the NHCl hydrolysates.
4. The 6 primary/secondary hydroxyl groups acetylated using acetic anhydride would correspond to the remaining hydroxyl functions available for acetylation on the glucose residues.

The amino acids detected in the subfractions are the same 14 as were found in the detergent solubilised membrane fraction from S. flava although present in different relative proportions.

The amino acids detected in the subfractions were also present in whole-number molar ratios which is indicative of a repeating unit of peptide within the peptide/protein from which they were derived.

Little can be said at this stage regarding the identity of the amino acid or the nature of the linkage involved in the bond between the peptide and glucose. It does seem unlikely however, that an O - serine or O-threonine linkage is involved since this would not be expected to withstand the strongly alkaline conditions of the saponification procedure used in the preparation of the pigment subfractions (Neuberger, Gottschalk & Marshall, 1966).

When the amino acid content of the subfractions is considered in relation to the content of carotenoid and glucose on a molar basis, it is at once apparent that these figures allow for the presence of a maximum of only 4 amino acid residues per carotenoid glycopeptide molecule. Thus it is obvious that each molecule cannot contain all 14 of the amino acids detectable by autoanalysis. It is suggested, therefore, that each subfraction, although chromatographically homogeneous in the system used, is in fact a mixture of molecules of similar basic structure but varying in the number and/or nature of the amino acids attached to the glucose molecules. From amino acid analysis, the relative proportions of the amino acids in the two subfractions was not the same which must account for their resolution. If, as the results of amino acid analysis suggest, there is a repeating unit of peptide in the protein layer of the membrane, and if the

distance between adjacent carotenoid molecules is less than the length of this repeating unit, then the latter must be fragmented during the isolation procedure. This would release glyco-carotenoid to which only portions of the unit would be attached and only when a mixture of these fragments was studied would this repeating unit become apparent. It is believed that such a situation is reported in this work.

In the light of the model proposed for the orientation of carotenoid within the membrane of S. flava it is perhaps relevant to consider here the effect of light on the growth and lysis of cultures of these cells. As can be seen from Fig. R.1. dark grown cells of S. flava, which were shown to contain 20% less carotenoid in their membranes, were also far less stable to lysis than the light-grown cells. The proposed arrangement of carotenoid molecules in the membrane would tend to confer a certain degree of rigidity and strength due to the covalent bridging of the two protein layers, in much the same way as di-sulphide cross-linking in proteins confers rigidity on the macromolecules. It is postulated that in the dark-grown cells, where 1 carotenoid molecule in 5 is missing from the membrane and replaced by other molecules e.g. phospholipids, metastable areas of the membrane result at which the mechanical strength of the membrane is weak and which become lysis points. Although this hypothesis is an attractive one, in that it substantiates the observations of Salton et al. (1965a,b) on the reduced stability of diphenylamine treated cells of S. lutea and M. lysodeikticus, it must be considered that the absence of light may

have effects other than carotenoid depletion which may be responsible for the greater fragility of the dark-grown cells.

The aqueous pigment fraction from S. morrhuae is a monodisperse system of molecules, if not a single molecular species, of approximately 9,000 molecular weight, containing carotenoid, glucose and peptide. As would be expected of a water soluble fraction, the protein content is considerably higher than that found for the methanol soluble carotenoid glycopeptide subfractions isolated from S. flava. Once again this material is believed to represent one form at least in which carotenoid is bound in the bacterial membrane. The linkage between pigment and glucose is again in all probability a glycosidic bond and it is assumed, as for S. flava, that in S. morrhuae it is the most polar free carotenoid which becomes bound to glucose and peptide and incorporated into the bacterial membrane. The molar ratio of glucose to carotenoid for this material was also found to be 2:1 and if its orientation within the membrane is also analogous to that suggested for S. flava, then two of the four tertiary hydroxyl groups on the bacterio-ruberin molecule (the most polar free carotenoid) would be involved in glycosidic links with glucose, at either end of the molecule.

The amino acids constituting the peptide moiety were not present in defined whole number molar ratios suggesting that in this case they are not derived from a peptide containing a repeating unit. Four small unidentified peaks were found on the amino acid analyser read-out. That which was seen between

phenylalmine and ammonia may correspond to ethanolamine and an unidentified component which appears in the same position has been found by the author in hydrolysates from S. flava membranes, and also by Grula et al. (1967) in hydrolysates from M. lysodeikticus membranes. The high content of acidic amino acids in the material is consistent with the observations of others of a high aspartic and glutamic acid content in the ribosomal proteins (Bayley, 1966) and the proteins of the cell envelope (Kushner & Onishi, 1966) of several species of halophilic bacteria. The rather high ash content recorded for the material is interesting in that no further salt was removable by pressure dialysis, so that this remaining salt must be tightly bound. It is presumed from the observations of Larsen (1967) concerning the salt requirements of halophilic bacteria, that much of this salt may be in the form of K^+ and perhaps Na^+ counterions associated with the large amounts of aspartic and glutamic acids present. Many of the proteins of obligately halophilic microorganisms show an absolute requirement for salt in order to maintain their structural and/or functional integrity. Many of the enzymes require salt for their activation, and the protein components of both the ribosomes and the cell envelope dissociate in the absence of salt (Larsen, 1967).

The high proportions of the acidic amino acids in the peptide, whose side chains would be negatively charged at physiological pH, accounts for the migration of the material on electrophoresis. In contrast, the detergent-solubilised material from S. flava contains few polar amino acid residues and such acidic

amino acids as are present are probably predominantly in the amide form, so that the peptide moiety would be expected to carry little net charge as was indicated by its lack of mobility on electrophoresis and the unsatisfactory titration behaviour. Also, the possibility exists that such ionisable side chains as are present are masked by the inclusion of the peptide moiety in a detergent micelle.

From this work on the bound carotenoid in both S. flava and S. morrhuae, it is suggested that the carotenoid biosynthetic sequence in both is directed towards the complexing of the most polar free carotenoid to glucose and peptide and its incorporation into the bacterial membrane as an integral structural component. If this is the case, then it is not surprising that for both species it is impossible to remove all the carotenoid pigment by simple solvent extraction since this would not be permitted by virtue of its covalent attachment to the protein of the membrane. In species such as M. radiodurans (Thirkell, 1968), S. aureus (Hammond & White, 1970) and F. dehydrogenans (Weeks et al., 1967), whose carotenoids can be totally extracted using organic solvents leaving a white residue, it seems unlikely that a similar sort of binding occurs between carotenoid and other membrane components

B. THE CHEMICAL COMPOSITION OF THE TOTAL MEMBRANE FRACTION FROM S. FLAVA

The yield of the total membrane fraction from S. flava decreases quite markedly with age, the decrease being most pronounced between 24 and 48 hours. This may well reflect an

increased amount of intracytoplasmic membrane in the 24 hour cells which are in the late exponential phase of growth, since, whether mesosomes are indeed involved in respiratory chain activity, septum formation, nuclear segregation, or are merely a means of increasing the total membrane surface area and hence the enzymatic content of the cell (Ryter, 1969), it would be expected that actively dividing cells might contain a greater amount of mesosomal membrane than cells which have entered the stationary phase of growth. Alternatively, these observations may indicate a thickening of the cell wall with age such that the contribution of the membrane fraction to the total dry weight of the cell is decreased. All three values for membrane content are relatively high when compared with the yields of membrane obtained from some other species, as shown in Table D.1. (overleaf). It should be noted that, from the table, S. lutea does exhibit the highest content of membrane of the organisms shown so that, in view of the results obtained here a high content of membrane may be a characteristic of Sarcinae. It seems unlikely that the high values obtained for S. flava could be due to cell wall contamination since cell wall components were not detected during the carbohydrate and amino acid analysis. Salton (1967b) has, in fact, found that cells of S. lutea contain a greater percentage of membrane at 48 hours than at 24 hours, although in an earlier paper (Salton et al., 1965b) it was stated that in some cultures of M. lysodeikticus, the proportion of membrane increased with age, but in other cultures the proportion was not found to vary significantly with age at all. Thus it seems possible that minor

Organism	Age	% membrane	Reference
B. licheniformis	24 hr.	10.6	Salton 1967b
B. stearothermophilus	9 hr.	16.2	Salton 1967b
M. lysodeikticus	24 hr.	25.2	Salton 1967b
S. lutea	24 hr.	25.0	Salton 1967b
S. lutea	48 hr.	31.0	Salton 1967b
M. lysodeikticus	20 hr.	8.6	Gilby et al. 1958
B. subtilis	18 hr.	28.0	Bishop et al. 1967b
L. monocytogenes	?	9.2	Ghosh et al. 1968
S. aureus	Late log phase	9.7	Ward et al. 1968
S. flava	24 hr.	49.6	Table R.11.
S. flava	57 hr.	36.8	Table R.11.
S. flava	91 hr.	34.5	Table R.11.

Table D.1. - Percentage of dry cell weight constituted by the total membrane fraction for several Gram-positive bacteria.

differences in culture conditions or species differences may have marked effects on the percentage of membrane synthesised by the bacterial cell.

In comparison with published figures for analyses of other Gram-positive bacterial membranes (see Table I. III.), the carbohydrate content of S. flava membranes is somewhat higher and the lipid content rather lower than those encountered in other species The remaining components, protein, RNA and total phosphorus were present in the same sort of percentages as those observed for the majority of Gram-positive organisms. It can be seen from Table R. 19., that the overall recoveries of organic material from S. flava membranes decreases with age, being 100.2% for the 24 hour preparation, 98.3% at 57 hours and only 82.5% for 91 hour membranes. In view of the difficulties experienced in achieving a total lipid extraction from the membranes, a possible interpretation for this decreased recovery might be an increase in the binding of lipid to protein in the membrane with age, which would result in an anomalously low value for the lipid content of older membranes. For many bacterial membranes e.g. B. subtilis (Bishop et al., 1967b) it is possible to extract virtually all the lipid using solvent extraction, since these workers found that negligible amounts of lipid were released by subsequent acid hydrolysis. However, as can be seen from Table R. 14., the amount of lipid which is released by hydrolytic procedures from S. flava membranes exceeds considerably that which is extracted by simple solvent extraction, indicating that the bulk of the lipid is firmly bound within the membrane.

Using the carotenoid pigments of the membrane as "markers" it was evident that the methods of extraction employed in this work did not by any means extract all the lipid from the membrane. Treatment with strong alkali did render the membrane almost totally soluble but this procedure would undoubtedly convert a large proportion of the lipid into water soluble degradation products. The results of lipid extractions from S. flava membranes (Table R. 14.) indicate a significant decrease in lipid content of 91 hour membranes but in fact as already discussed it is possible that this merely reflects an increased binding of lipid to protein with age. This would mean that either the percentage of the total lipid which is extractable decreases or the proportion of the total lipid which is extracted by the hydrolytic procedures, and hence the losses sustained due to degradation, might increase thus giving an anomalously low lipid content. Furthermore, it has already been shown in this work that a substantial amount of the carotenoid pigment is covalently linked to protein in the membrane, and work at present in progress suggests that much of the membrane phospholipid is also linked to protein in this manner, so that, an increase in the proportion of the total lipid so bound with age could account for the findings presented in this work.

Although the molar ratios of the amino acids present in the membrane remain fairly constant, the overall protein content decreases with age. This conflicts with the findings of Salton et al. (1965b) for the protein content of M. lysodeikticus

membranes which was found not to vary significantly between 24 and 48 hours. The acidic and apolar amino acids are present in much greater concentrations than either the basic or sulphur-containing amino acids, which is consistent with other data (see Introduction) for the amino acid composition of bacterial membrane proteins. The detection of a component which may be glucosamine on amino acid analysis, but the absence of any trace of glucosamine during the paper chromatographic analysis of membrane carbohydrates may be explained by the more vigorous hydrolytic conditions employed for the release of amino acids, since the N HCl hydrolysis used for carbohydrate release may not release sufficient amounts of the amino sugar to be detectable by the latter technique.

The carbohydrate content of the membrane fraction remains fairly constant with age, as do the relative proportions of the individual monosaccharides. Glucose, mannose, ribose, and rhamnose have been shown to be definitely present, the former two occurring as major components. Indications were also obtained from paper chromatography and amino acid analysis for the presence of galactosamine and glucosamine. The ribose detected is almost certainly derived from the RNA component of the membrane. Glucose has already been shown to be associated with carotenoid and may well be a component of other glycolipids or of glycoproteins in the membrane, whilst mannose may also be a glycolipid component since the closely related species M. lysodeikticus is known to synthesise considerable amounts of a

mannosyl diglyceride (MacFarlane, 1961). The occurrence of rhamnose in a bacterial membrane is comparatively rare, and at the time of writing only two other reports of this exist, one for *L. monocytogenes* (Ghosh *et al.*, 1968) and one for *Bifidobacterium bifidum* (Exterkate, Vrensen & Veerkamp, 1970). Galactose, although reported to be a component of the membranes of both *S. lutea* and *M. lysodeikticus* (Salton *et al.*, 1965b) was not detected in *S. flava* membrane preparations by either GLC or paper chromatography.

The RNA and total phosphorus content of the membrane show some variation with age. The significance of the fluctuation in RNA content is not clear since this was found to decrease from 24 to 48 hours, and then at 91 hours to increase to a value intermediate between the 24 and 48 hour values. This slight rise in RNA content at 91 hours presumably indicates increased protein synthetic activity, which, in view of the age of the cells may possibly be connected with the production of autolytic enzymes or with the onset of sporulation, which the ultrastructural study presented in this thesis has suggested may occur in *S. flava*. The total phosphorus content was found to decrease with age and it is thought that this might reflect a reduction in the phospholipid content of the membrane, although it has been found by Ghosh *et al.* (1968) that the RNA and lipid of the membrane of *L. monocytogenes*, by no means account for the total phosphorus content, some of which may also be derived from teichoic acid and phosphoserine.

S. flava, in common with many other bacterial species, synthesises a large number of different fatty acid types, including a high proportion of branched chain compounds. Bishop et al. (1967b) have reported that branched fatty acids constitute more than 75% of the total fatty acids in the membrane of B. subtilis. The branched C_{15} acid which was found to be the major component of the fatty acids of the free lipid fraction from S. flava membranes has also been reported to be the predominant fatty acid in the lipids of the following species : C. acnes (Moss et al., 1967), several Propionibacteria (Moss et al., 1969), S. lutea (Cho et al., 1966, Tornabene et al., 1967, Albro et al., 1969), M. lysodeikticus (Cho et al., 1966), S. aureus (White et al., 1968) and several Bacilli (Kaneda, 1967, 1968). The dearth of unsaturated fatty acids detected in any of the lipid fractions from S. flava membranes is also a feature common to many Gram-positive bacterial lipids (Kaneda, 1963; Tornabene et al., 1967; Bishop et al., 1967b; Moss et al., 1969). It can be said with reasonable certainty that the unidentifiable fatty acid components detected by GLC are not unsubstituted straight chain or branched compounds but probably possess hydroxyl or ring substitutions which have been reported as components of the fatty acids of a variety of bacteria (Cho et al., 1966; O'Leary, 1962).

The considerable variation in the fatty acid composition of the membrane fraction from S. flava with age conflicts with the observations of Tornabene et al. (1967a) on the fatty acid composition of S. lutea, which was found not to alter significantly with age. Nevertheless, the fact that such a variation was

observed, as shown in this work, infers that for S. flava anyway, the precise stage of the growth cycle at which the bacterial culture is harvested will dictate the observed fatty acid pattern. This finding would make the use of the fatty acid profile as a taxonomic criterion of dubious value for this species.

C. ULTRASTRUCTURAL FEATURES OF S. FLAVA AND S. MORRHUAE

1. S. flava

It was found that the appearance of thin sections of S. flava cells varied markedly with the conditions of fixation. Double fixation with glutaraldehyde/osmic acid (OsO_4) was unsuccessful at any of the three pH values, owing to the deposition of fine needle-like crystals over the entire specimen, obscuring any fine structure which might have otherwise been apparent. Therefore, no electron micrographs of S. flava cells fixed under these conditions are included in the Results section. This crystalline deposit may well be due to the incomplete removal of the glutaraldehyde prior to post-fixation with osmium, since Gordon, Miller and Bensch (1963) recommended that material which had been fixed with glutaraldehyde should be washed for several hours with buffer before being fixed with OsO_4, whereas the glutaraldehyde fixed cells in this work were washed twice, over a period of only one hour. A longer period for washing was not employed since one hour had proved adequate for the fixation of S. morrhuae cells by exactly the same method.

Fixation with $KmnO_4$ gave best definition at pH 8 (Plate 3), where the preservation of the cytoplasmic membrane is good although the highly oxidative conditions can be seen to have

caused much greater disruption of cell wall material than fixation of similar material using OsO_4. Cell wall debris can also be seen surrounding the cells in Plates 1 & 4. Permanganate fixation at pH 6 (Plate 4) gave poor definition generally, the cytoplasmic membrane being indistinct and the nuclear material having a vacuolated appearance which can also be seen in Plate 3. Treatment with the same fixative at pH 4 (Plate 1) gave a coarse, granular appearance to the cytoplasm, although in this case the cell wall material appears relatively intact. Neither cytoplasmic membrane nor nuclear material are visible and both cells have undergone a considerable degree of plasmolysis. Thus fixation at alkaline pH appears to give the best results for permanganate fixation, although the only ultrastructural feature to be well preserved at all by this method is the cytoplasmic membrane, and even the preservation of this is not superior to that obtained using osmium fixation.

Examples of cells fixed with OsO_4 at pH 9 are shown in Plates 25, 27, 28 & 31-34. These cells show a dense, granular cytoplasm, a thick amorphous cell wall, devoid of fine structure and in some cases (Plates 28, 31 & 32) ribosomes are visible. In Plates 28 & 31-33 the membrane material has a negatively stained appearance i.e. two electron-translucent layers separated by an electron-dense layer. Nuclear material is not clearly distinguishable in any of these cells and is probably obscured by the density of the cytoplasm.

Osmium fixation at pH 4 and subsequent double staining

with uranyl acetate/lead citrate (Plate 2) gave very poor general preservation. The cytoplasm is made up of dense, granular aggregates and neither membrane nor nuclear material are distinguishable.

Excellent definition of membrane, cell wall and nuclear material was given by osmium fixation at pH 8, followed by double staining (Ur/Pb). Examples of cells treated thus are shown in Plates 5-21. The improved appearance of these cells over those fixed with osmium at pH 9 may not only be due to the difference in pH but also possibly to one or more of the following factors:-

(i) increased fixation time (16 instead of 4 hrs.)

(ii) inclusion of a prestaining step with uranyl acetate

(iii) inclusion of KCN in the fixative fluid (Highton, 1969)

(iv) double staining with Pb/Ur as opposed to single staining with Ur alone.

During the course of this work, several hundred sections of lysozyme-treated cells were examined, but only on a few occasions were true protoplasts observed, totally devoid of any adhering cell wall material. Plate 19 shows a protoplast about to be released from the remnants of the cell wall. Note the considerable reduction in the density of the cytoplasm of this cell as compared with others still possessing the majority of their cell wall, suggesting that here, although the cytoplasmic membrane appears to be intact around the complete circumference of the cell, lysis may have occurred in another plane. From

the appearance of the majority of the lysozyme treated cells, it must be concluded that the method for the preparation of protoplasts used in this work (Baird-Parker *et al.*, 1967), although claimed to be suitable for *B. megaterium* and *M. lysodeikticus* is not applicable to *S. flava*. Apart from an increase in the incubation time, which anyway would be expected to ensure a more complete digestion of the cell wall, the conditions as detailed in the published method were adhered to rigorously. Thus it must be concluded that the cell wall of *S. flava* is more resistant to the action of lysozyme than that of either *B. megaterium* or *M. lysodeikticus*.

The possibility must be considered that, although protoplast formation was evidently not complete, those which were formed might have been destroyed by the conditions employed in the preparation for electron microscopy. Indeed, Kellenberger & Ryter (1964) have stated that osmium fixation may cause immediate lysis of protoplasts, although Fitz-James (1964) has published excellent electron micrographs of protoplasts fixed with osmium. Moreover, if massive lysis of protoplasts had occurred, one would expect to find large amounts of cytoplasmic debris and membrane fragments, which were not in fact observed in these preparations.

With regard to these findings, it would seem advisable, if applying a method for protoplast or membrane preparation to a species for which it was not originally described, no matter how closely related, to ensure, preferably by electron microscopy, that complete removal of all cell wall material has occurred.

However, as mentioned earlier, indications of cell wall contamination can also be obtained during chemical analyses of membrane preparations.

The general ultrastructural features of S. flava cells are similar to those observed for many other Gram-positive bacteria. The cells vary in diameter from about 0.5-1.5 μ and pairs of cells were observed most frequently although tetrads (Plates 2,4,5,11,14 & 16) and occasionally larger groups (Plate 20) were seen. Ribosomes are visible in many cells (Plates 22, 25,28,31 & 32) as granules approximately 100-200 Å in diameter. Usually these ribosomes were seen uniformly distributed throughout the cytoplasm but in certain cases (Plate 28) they were concentrated in the region of developing septa and probably also in association with dividing nuclear material, as was shown for D. pneumoniae (Tomasz et al., 1964), although in this cell the nucleus is not distinguishable. Many of the cells fixed with OsO_4 at pH 9 were found to contain large (800-1000 Å dia.), circular, darkly staining bodies with smaller, lighter areas at their peripheri (Plate 33). These are thought to be identical with the polymetaphosphate granules which have been described in several other Gram-positive organisms (Widra, 1959; Thornley et al., 1965; Cherny, 1967; Holt et al., 1967; Hard, 1969). Friedberg et al. (1968) have isolated and analysed a fraction containing granules of similar morphology from M. lysodeikticus finding it to consist of 24% protein, 30% lipid and 27% phosphate. These workers found that the granules were most abundant during

the exponential phase of growth, disappearing gradually during the stationary phase. This might explain why no such inclusions were observed in cells of S. flava grown for 60 hours.

The nuclear material of most of the lysozyme treated cells, fixed with osmium at pH 8, (Plates 5-8, 12-14, 17, 20 & 21), shows the dense fibrillar arrangement which is now accepted as being characteristic of well-fixed DNA. In addition to this parallel arrangement of fibrils, in some sections (Plates 5 & 7) these are interspersed with small, dark, circular granules which are much too small to be ribosomes and may well be transversely sectioned fibrils of DNA. In most of these cells, the nucleus is a single, fairly compact body, whilst in some (Plates 5 & 8) it occupies a large proportion of the cytoplasm at the particular plane of the cell which has been sectioned. In other cells (Plates 7 & 14), the nuclear material can be seen in two or three separate portions although again, this may be an effect due purely to a fortuitous plane of sectioning.

Plates 5,8,12,17, & 20 show (arrowed) possible areas of contact between nuclear material and the cytoplasmic membrane. It has long been known that in many species of bacteria there is an intimate association between the nucleus and mesosomal or cytoplasmic membrane. Jacob, Brenner & Cuzin (1963) first proposed that the bacterial nucleus and the mesosomal membrane were attached by a serial sectioning study of B. subtilis. The exact point of attachment is difficult to define in B. subtilis, since the area of contact between membrane and nucleus is often extensive, but when mesosomes are extruded from the cytoplasm

during protoplasting (Ryter *et al.*, 1964), the nucleus is pulled up to, and remains in contact with, the cytoplasmic membrane, when the exact point of attachment may be distinguished more clearly. Similarly, the nucleus can be seen to be directly in contact with the cytoplasmic membrane in reverted or reverting protoplasts (Ryter & Landman, 1967). Attachment of the nuclear material to membranous material has since been observed in many species other than *B. subtilis*, including : *S. lutea* (Cherny, 1967); *S. aureus* (Suganuma, 1968); *E. coli* (Bayer, 1968a; Pontefract, Bergeron & Thatcher, 1969; Pontefract & Thatcher, 1970); *S. lactis* (Thomas, Lyttleton, Williamson & Batt, 1969); *S. faecalis* (Higgins *et al.*, 1970) and *B. licheniformis* (Highton, 1970). It may be that in *S. flava* also the nucleus is always attached to membrane but that the actual point of attachment is not always seen due to an unfavourable plane of sectioning. The connection between mesosomes and nuclei in bacteria has led to the postulate that mesosomes may be involved in the segregation of the nuclear material during cell division, (Ryter & Jacob, 1967) Although this may be the case for some cells, the participation of mesosomes in nuclear segregation cannot be obligatory since protoplasts and other cells devoid of mesosomes are nevertheless still capable of division.

Intracytoplasmic membranous inclusions were seen in many of the thin sections of *S. flava* cells studied. Some or all of these may be identical with, or derived from, mesosomes. The appearance of a mesosome under the electron microscope, which

most nearly represents its morphology in the true physiological state is much disputed. In some cases, mesosomes are seen as predominantly lamellar structures consisting of either concentric rings of membrane or a more complex "swiss-roll" arrangement. Both these lamellar formations are seen in B. licheniformis (Highton, 1970a). Other workers (Tomasz et al., 1964; Ryter, 1968) have shown mesosomes to be membranous pockets enclosing large numbers of membrane-bound vesicles by normal thin-sectioning techniques, whilst others (Remsen, 1968; Nanninga, 1968) have also confirmed the presence of vesicles within mesosomes by freeze-etching studies. In addition, several reports also exist of mesosomes in the same bacterial cell containing both lamellar and vesicular elements (Thomas et al., 1969; Highton 1970a,b; Neale & Chapman, 1970; Higgins et al., 1970). Further support for the vesicular structure comes from observations on mesosomes extruded from protoplasts. Incubation of B. subtilis (Ryter & Landman, 1964) or L. monocytogenes (Ghosh & Murray, 1967) cells in hypertonic medium, results in plasmolysis of the cells and the extrusion of their mesosomes such that the mesosomal elements come to lie in the periplasmic space, between the wall and the membrane. Subsequent removal of the cell wall by treatment with lysozyme results in the pinching off of the mesosomal tubules which are thus released into the medium. Both before and after lysozyme treatment, these mesosomal tubules have the appearance of a chain of small vesicles (the so-called "string of beads" structure). Ryter (1969) thus concludes that the

true structure of a mesosome is the vesicular form and that this structure may assume a lamellar appearance only if sectioned in an unfavourable plane or if the specimen has been fixed with osmium, which it is said transforms the vesicular form into the lamellar form.

Conversely, Highton (1969, 1970a,b) has presented evidence that lamellar form is the true physiological morphology for a mesosome and that the vesicular mesosomal elements are formed from the breakdown of the lamellae. This was found to occur if B. subtilis cells were allowed to stand for some time after harvesting, at 0^{o}, prior to fixation, after which they were found to contain many vesicular mesosomes. Cells fixed immediately after harvesting exhibited mesosomes with a completely lamellar appearance. Recent work has also shown that shifts in growth temperature (Neale et al., 1970) and starvation (Thomas et al., 1969; Higgins et al., 1970) of bacterial cells cause the breakdown and vesicularisation of mesosomes, sometimes accompanied by their extrusion.

In S. flava cells which had not received lysozyme treatment, lamellar mesosomes were readily visible in many cells, whose appearance corresponded to those shown in Plates 28 & 32. In both cases the mesosomes may be seen to consist of complex whorls of membrane, continuous with the cytoplasmic membrane. In all cases, where mesosomes were observed, they were never associated with developing septa, but usually situated at the poles of a dividing cell.

Lysozyme treated cells contained few lamellar mesosomes but many contained membrane bound vesicles, enclosing small portions of cytoplasm. An example of one of the few lamellar structures is shown in Plate 12., where the membrane is coiled into the "swiss-roll" type of arrangement mentioned previously, and can be seen to be continuous with the cytoplasmic membrane. Plates 5,10,11,14,16,17 & 20 show intracytoplasmic vesicles consisting of a single layer of membrane enclosing a portion of cytoplasm, but with an electron translucent gap between the enclosed cytoplasm and the membrane. Plate 13 shows a similar vesicle consisting of two concentric rings of membrane enclosing a small area of cytoplasm. In many instances (Plates 5,13,14,16) fine strands of material connect the enclosed cytoplasm and the enveloping membrane. Some cells (Plates 10,15 & 20) show membrane bound vesicles lying in the periplasmic space i.e. between the cytoplasmic membrane and the cell wall, and these were also occasionally observed in cells which had not been treated with lysozyme, although these cells were usually seen to be in the process of lysis, as is shown in Plate 27. An extra piece of membrane (arrowed), which does not appear to be connected to the cytoplasmic membrane can be seen in the periplasmic space of the cell shown in Plate 11. It is suggested that these membranous inclusions in the periplasmic space, and all the membrane bound vesicles seen in the cytoplasm are derived from the breakdown of mesosomes under the hypertonic conditions (0.75 M sucrose) employed in the attempted preparation of protoplasts.

It has already been shown that under hypertonic conditions mesosomal extrusion occurs in some cases, into the periplasmic space but other reports (Landman, Ryter & Frehel, 1968; Neale *et al.*, 1970) have shown mesosomal remnants which have been released into the cytoplasm as is believed to be the case in this work. A partially lysed cell containing numerous membranous vesicles, one of which consists of two concentric rings of membrane, is shown in Plate 9. This double membrane vesicle is not unlike that shown in a partially lysed cell of *D. pneumoniae* in the report of Tomasz *et al.*, (1964). These workers suggest that these vesicles are once again of mesosomal origin and this is also probably the case for *S. flava*.

The cell wall of Gram-positive bacteria has generally been considered to be thick, uniform, amorphous and devoid of fine structure although "a suggestion of a multi-layered appearance has been observed in some cases" (Salton 1964). More recent work has shown that, although in some species the cell wall appears to be amorphous, e.g. in *S. maxima* & *S. ventriculi* (Holt *et al.*, 1967); *S. lactis* (Thomas *et al.*, 1969); *S. morrhuae* (Brown *et al.*, 1970), in many other Gram-positive bacteria the cell wall exhibits a laminated appearance. A trilaminated cell wall, consisting of two thin electron opaque lines, separated by a rather wider less dense layer, somewhat reminiscent of a unit membrane although less well defined, has been reported in *B. subtilis* (Highton, 1970b; Neale *et al.*, 1970) *B. licheniformis* (Highton, 1970a); *S. faecalis* (Higgins *et al.*, 1970) and *S. aureus* (Suganuma, 1966, 1968). Hughes, Tanner &

Stokes (1970) have shown that thickening of the cell wall of B. subtilis caused by tryptophan deprivation, results in the production of a 5-layered cell wall structure after only 30 mins. incubation in a medium lacking the amino acid.

Intact cells of S. flava fixed with OsO_4 (Plates 25-28 & 31-34) show a typical amorphous unlayered cell wall, but cells fixed with $KMnO_4$ (Plates 24,29 & 30) display a distinctly laminated cell wall, consisting of a compact basal layer of electron dense material adjacent to the cytoplasmic membrane, separated from an outer band of similar density although a little less-well defined, by a broader layer of less dense material. This arrangement is rather like the trilaminar appearance already described for other cell walls.

In cells of S. flava where partial digestion of the cell wall due to the action of lysozyme has taken place, a multi-layered structure for the cell wall is much more apparent. Three cell wall layers are discernable in the cells shown in Plates 5,8,11,13,14,15 & 20, whilst Plate 18, although atypical shows a damaged cell in which four electron dense layers can be seen to constitute the cell wall. These layers are not continuous, however, being broken at two points, the cell itself having partially lysed. Plates 4,5,7,11,14,16 & 20 show packets of cells which appear to be held together by a strand of lysozyme resistant cell wall material which runs around the outside of just a pair of cells (Plates 14 & 20) or envelopes the whole packet (Plates 4,5, 7,11 & 16). Spaces are visible between adjacent layers of the cell wall in almost all cases, which have presumably arisen from

the complete digestion of selected portions of the wall which are lysozyme-sensitive, leaving behind these electron dense layers which thus become much more obvious and must be rather more resistant to the action of the enzyme. These lysozyme resistant layers must be regions of the wall where the glycan backbone is in some way stabilised against or protected from lysozyme attack. Perhaps the degree of peptide cross-linking between the polysaccharide chains in these regions is greater than in the lysozyme sensitive areas. The stratification of the cell wall might be brought about by the laying down of successive layers of peptidoglycan as the cell grows and the wall expands, each layer differing chemically, albeit very slightly, from the next. Another facet of cell wall structure is shown in Plate 2, which shows what has obviously been a tetrad of cells that have become separated, but are still connected by fine threads of cell wall material. This type of appearance was only observed for cells fixed with OsO_4 at pH 4. At pH 8 as already indicated the groups of cells are held together by an outer layer of lysozyme resistant material and it is conceivable that under the acid conditions of fixation at pH 4 that this layer is further broken down allowing the separation of the cells.

Owing to the hypertonicity of the sucrose solution in which protoplast formation was attempted, all the lysozyme treated cells examined were plasmolysed to some extent. In Plates 8,14, 17 & 20, it can be seen that, although the cytoplasmic membrane has retracted from the cell wall, portions of the membrane are still in contact with the wall. This is particularly apparent

in the cell shown in Plate 8, where the regions of adhesion between wall and membrane are arrowed. Connections between cell wall and cytoplasmic membrane have been described for a number of other microorganisms such as S. coelicolor (Glauert & Hopwood, 1960); B. subtilis (Glauert, Brieger & Allen, 1961); L. monocytogenes (Edwards & Stevens, 1963) and E. coli (Bayer, 1968a). Bayer (1968a) suggested that in E. coli these points of contact between wall and membrane might be centres of cell wall synthesis and that these might correspond to mechanically weak areas which would become lysis points, were the cell subjected to osmotic stress. These regions of adhesion have also been shown (Bayer 1968b) to be the points at which the bacteriophages T_1 - T_7 are adsorbed when attacking the E. coli cell. If the attachment at these points is fairly stable, this would make the preparation of wall and membrane fractions which were without any mutual contamination somewhat difficult, as has been found by Salton (1967d). Bayer (1968a) also reports the association of nuclear material with these membrane-wall junctions, but no such association was discernable in any of the S. flava cells examined in this work. More information concerning the nature and stability of the junctions in S. flava could be obtained by study of cells which have undergone a greater degree of plasmolysis.

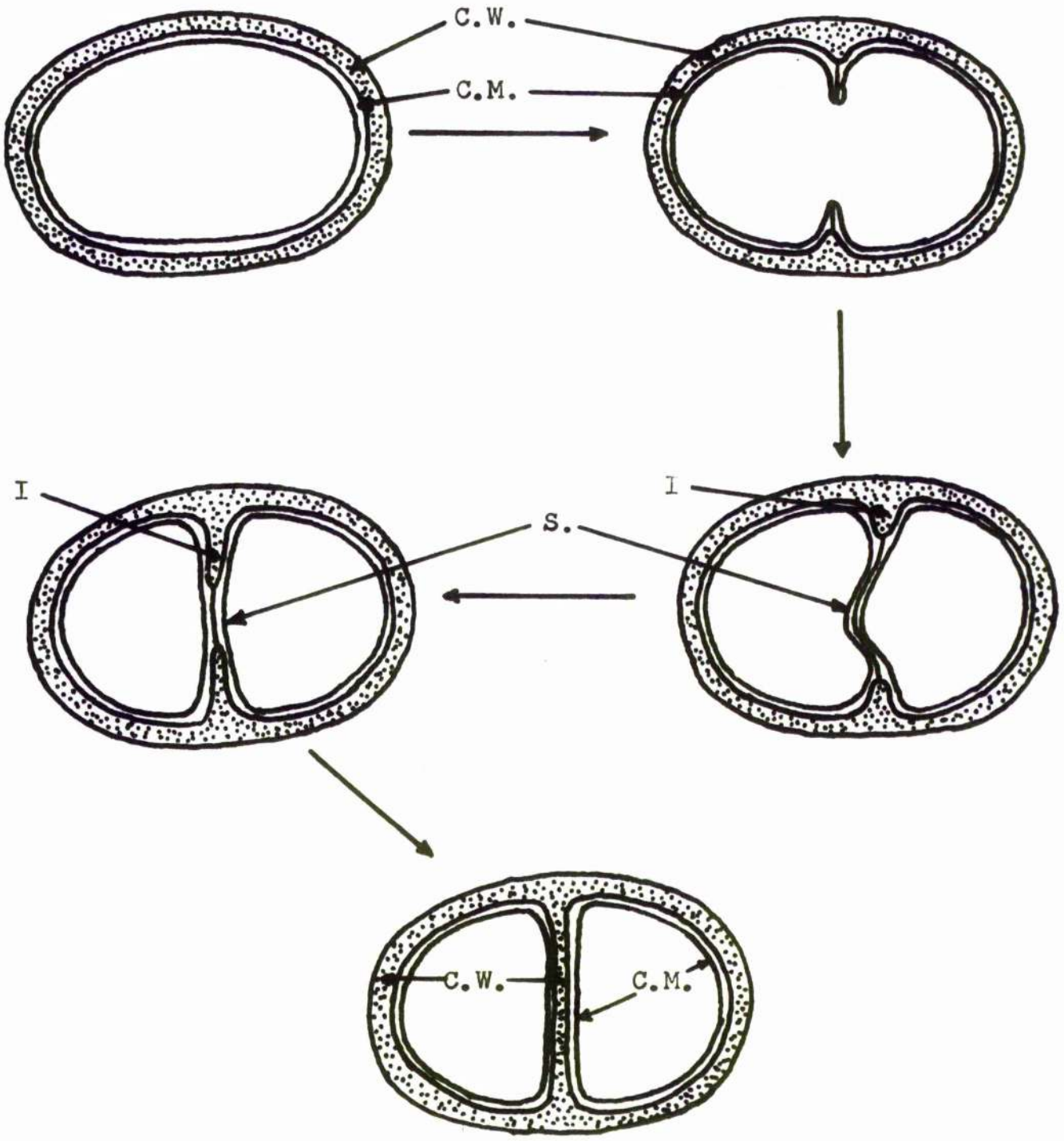
Though some Sarcinae are known to form spores (e.g. S. ureae) sporulation in S. flava has never been previously reported. In several of the preparations examined in this work, however,

spore-like bodies with very thick, amorphous walls enclosing a highly granular cytoplasm were seen. Two such bodies are shown in Plates 22 & 23 and a distinct cytoplasmic membrane and numerous ribosomes are visible in each. Also, in the preparations of lysozyme treated cells, several cells resembling those shown in Plate 21 were seen. In the left-hand cell, the nuclear material is surrounded by an unusually dense region of cytoplasm, around which can be distinguished a layer of membrane. Outside this can be seen what appears to be a double layer of cell wall material in the process of formation. In the cell on the right, the same process seems to be occurring, although not at quite such an advanced stage. These cells, it is postulated, are undergoing sporulation and from these observations it seems that, under certain adverse conditions e.g. in the presence of sub-lethal concentrations of lysozyme, S. flava cells may be induced to form spores. The likelihood of the spore-forming cells being contaminants of the culture seems remote since: (i) many spore-like bodies and cells of similar appearance to those shown in Plate 21 were seen; (ii) the cells which appeared to be sporulating possessed the same basic ultrastructural features as the remaining cells in the cultures.

A chronological reconstruction of the events involved in cell division in S. flava is shown in the sequence Plates 28 - 34. In the cell shown in Plate 28, the cell wall is beginning to grow inwards at its centre, and a membranous projection can be seen extending from the tip of the uppermost cell wall extension. The rate of synthesis of membrane material now appears to exceed

that of cell wall, so that membranous projections extend further into the cytoplasm, as shown in Plates 29 & 30. The growth of the membranous material is not always uniform as can be seen in Plate 30, and even more strikingly in Plate 17. In Plate 31, a membrane septum can be seen, which completely divides the cell. This membrane septum appears to consist of a double membrane, which is again abundantly clear in the developing septum shown in Plate 17, each half of which is continuous with the cytoplasmic membrane on either side of the cell wall protrusions. Seemingly, cell wall material is then laid down between the layers of the double membrane septum, as shown in Plate 32, where the septum appears as a loop of membrane in the cytoplasm. The septation process is further advanced in Plate 33, where only a short length of membrane connects the two halves of the ingrowing cell wall septum. Plate 34, shows the division process completed, a continuous layer of cell wall material having effected the complete separation of the daughter cells.

This division sequence is represented diagramatically in Fig. D.2. When considered three-dimensionally, the double membrane septum must be a complete sheet of material which divides the whole cell, and which develops by annular growth of an infolding of the cytoplasmic membrane at the equator of the cell. The establishment of this membrane septum is then followed by the deposition of cell wall material, between the two layers of the double membrane, and continuous with the original cell wall, until a complete dividing septum of cell wall material has



C.W. - Cell wall

C.M. - Cytoplasmic membrane

I. - Ingrowing cell wall

S. - Double membrane septum

Fig. D.2. - Diagramatic representation of the proposed mode of cell division in S. flava.

been formed. In this way, each half of the double membrane septum becomes part of the cytoplasmic membrane of each daughter cell. The findings here show that partition of the S. flava cell by cell wall material is definitely preceded by the establishment of a complete membrane septum, and thus corresponds to the process termed "cell membrane septation" by Chapman (1959). Mesosomes were seen, but never in the region of developing septa, as has been described for so many other Gram-positive species (see Introduction). The division scheme proposed here supports the observations of Chapman (1959) concerning the mechanism of septum formation in an unidentified Gram-positive species and is also similar to that reported for D. pneumoniae (Tomasx et al., 1964).

2. S. morrhuae

Individual cells of S. morrhuae are almost twice as large as those of S. flava, being 2 - 4 m. in diameter. A low-power electron micrograph (Plate 35) shows the general appearance of a group of cells. Although pairs and tetrads were visible, as for S. flava, irregular clumps of cells were more common, indicating that cell division in this Sarcina species may well be more random process than in S. flava, where each successive plane of division is at right angles to the previous one, producing the cuboid packets of cells characteristic of the Sarcinae. An irregular type of cell division is shown in Plate 40, where the cell appears to be forming two septa simultaneously, which, it appears will eventually divide the cell into three.

Plates 36,41 & 43 show cells containing crooked cell wall septa, of variable thickness, in contrast to the straight septa of uniform thickness observed in S. flava. Cell division in S. morrhuae would appear to be of the cell wall septation type (Chapman, 1959; Chapman et al., 1953) since neither complete nor nascent membrane septa were observed.

Plates 35-37 & 41-44 show cells fixed with OsO_4, whereas those in Plates 38-40 are double-fixed with glutaraldehyde/OsO_4. It is surprising that, in the case of S. morrhuae double fixation does not produce the crystalline deposit which obscures the fine structure of S. flava cells fixed and stained in an identical manner. Both methods of fixation produced what is generally regarded as poor preservation of nuclear material, which appears (Plates 35-40) as darkly staining fibrous networks, of varying morphology, suspended in an electron translucent vacuole. These aggregates of nuclear material are particularly well defined in Plates 37 & 40, where they can be seen to be connected to the cytoplasm by fine strands, (arrowed). In Plates 39 & 40 the nuclear material is in the process of division and has assumed a typical "dumbell" configuration. The poor fixation of the nuclear material and possibly the lysis in some of the cells shown (Plates 36,38,39,41 & 43) may result from an inadequate content of salts in the fixation medium. No extensive ultra-structural studies have been carried out on Halococci up to the present time but exposure of cells of Halobacteria to media containing less than 4.3M NaCl results in the disintegration of

the cell envelope and membrane (Steensland & Larsen, 1969). Thus the fixation of S. morrhuae cells in higher concentrations of NaCl than those used in this work may improve the preservation of the ultrastructural features. The overall osmolarity of the fixative solution used here must, however have been high enough since many of the cells can be seen to have plasmolysed (Plate 44) In this and other plasmolysed cells, areas of adhesion between wall and membrane, as seen in S. flava, are not apparent.

The cell wall of S. morrhuae appears as a much more densely staining layer than that in S. flava, and assumes a very granular quality, particularly in those cells which were double fixed (Plates 38-40). In Plates 37,42 & 44, the wall can be seen to consist of three layers (arrowed) as already described for other Gram-positive bacteria, including S. flava. In other cells, this laminated appearance has been obscured due to the density and granularity of the staining. Another feature of the cell wall of S. morrhuae, not seen for S. flava, is the covering of its outer surface with fine fibrous projections. These are clearly shown in Plates 36,37 & 42-44. This outer covering may possibly be a polysaccharide coating, like the cellulose layer shown to envelope cells of S. ventriculi (Canale-Parola, Borasky & Wolfe, 1961) or may perhaps reflect the partial disintegration of the cell wall in response to the unphysiological salt concentration of the fixative solution.

No mesosomes as such were observed, but partially lysed cells, as shown in Plates 36,39 & 41, contained membrane bound

vesicles, which may well be derived from mesosomal membranes as previously described for S. flava. The general definition of membranes was not as good as that seen in S. flava but the typical unit membrane appearance was visible in most cases.

Many of the cells examined contained large numbers of electron dense, granular, spherical or ovoid bodies at their peripheri (Plates 38 & 44) which in most cases may be seen to be attached to the cytoplasmic membrane. In Plate 38, for example, several of these bodies clearly either interrupt or obscure the membrane. Plate 39 shows a cell containing a large aggregate of similarly staining material. The nature of these bodies has not been established, but they do not resemble the polyphosphate granules seen in other species, including S. flava. Their staining properties suggest that they are composed in part of either lipid or protein, but further work is necessary for their characterisation.

SUMMARY

SUMMARY

1. The preparation, purification and properties of a water-soluble membrane component from S. flava using the synthetic detergent Lubrol L has been described. This fraction contained carotenoid, glucose and peptide, was highly stable to heat and pH extremes, and release of free carotenoid from it proved extremely difficult. The possible effect of the binding of the membrane components within detergent micelles is discussed and the dangers inherent in the determination of molecular weights, by osmometry or ultracentrifugation, in the presence of detergent have been indicated.

2. The polar carotenoid subfractions from S. flava have been characterised and found to consist of carotenoid, glucose and peptide. The linkage between carotenoid and glucose is presumed to be glycosidic, and a model for the in vivo orientation of the carotenoid complex in the bacterial membrane has been proposed. A possible correlation between membrane stability and carotenoid content has been found, and this is discussed in relation to the model.

3. After complete removal of the free pigments from S. morrhuae by solvent extraction, a water soluble carotenoid fraction was isolated and characterised. The material, whose molecular weight is approximately 9,000, contains carotenoid, glucose and peptide. The bond between glucose and carotenoid is again presumed to be glycosidic, and the peptide moiety contains high proportions of the acidic amino acids, the signifi-

cance of which is discussed. This bound pigment is also thought to represent one form in which carotenoid is bound in the bacteria membrane.

4. The effect of the age of the culture on the chemical composition of the total membrane fraction from S. flava has been investigated. Both protein and lipid contents decrease with age although there is little variation in carbohydrate content. It is suggested that the decreased lipid content is a reflection of the increased binding of lipid to protein with age. Considerable variation in the fatty acid composition with age was observed, which makes the use of the fatty acid profile as a taxonomic criterion for this species of doubtful value. The presence of a sterol in S. flava membrane lipids is indicated although from GLC data, it seems unlikely that this is cholesterol. Monosaccharides detected in membrane hydrolysates were ribose, rhamnose, glucose, and mannose. The presence of glucosamine and galactosamine was also indicated.

5. The general ultrastructural features of whole cells of both S. flava and S. morrhuae have been described. S. flava exhibits many of the fine structural features common to Gram-positive organisms and was seen to form the packets of cells typical of the Sarcinae. Cell division in S. flava was shown to be of the cell membrane septation type, and a mechanism for this mode of division has been proposed. Preparation of protoplasts from S. flava using the method of Baird-Parker and Woodroffe (1967) was unsuccessful, but the treatment with lysozyme

revealed a layered appearance of the cell wall. Several intracytoplasmic membranous inclusions were seen in these cells, and their relationship to mesosomes is discussed. Mesosomes as such were also present, but never in association with developing septa. The effect of varying conditions of fixation on the fine structure of S. flava was also studied. Evidence has been presented that, under certain conditions, sporulation may occur in S. flava.

Good fixation of S. morrhuae cells proved difficult to achieve, and this may be due to insufficient concentration of salts in the fixation medium. Cells of S. morrhuae are approximately twice the size of S. flava cells, and division was seen to occur in a much more random fashion, producing irregular clumps of cells with common cell walls. Spherical or ovoid bodies of unknown composition were seen in association with the cytoplasmic membrane.

APPENDIX

APPENDIX

The following publications have already been produced from material included in this thesis:-

1. Thirkell D., Hunter, M.I.S., Crawford, J. & Fracassini, A.S. The effect of synthetic detergent on the determination of the molecular weight of a carotenoid-glycoprotein from S. flava. J. Gen. Microbiol. 56, 109-111, 1969.
2. Thirkell, D., Hunter, M.I.S. Carotenoid-glycoprotein of S. flava membrane. J. Gen. Microbiol. 58, 289-292, 1960.
3. Thirkell, D., Hunter, M.I.S. The polar carotenoid fraction from S. flava. J. Gen. Microbiol. 58, 293-299, 1969.
4. Thirkell, D., Hunter, M.I.S. A water-soluble carotenoid-glycopeptide from S. morrhuae. J. Gen. Microbiol. 62, 125-127, 1970.
5. Thirkell, D., Hunter, M.I.S. Cell division in S. flava. Biochem. J. 120, 13P, 1970.
6. Hunter, M.I.S., Thirkell, D. Variation in fatty acid composition of S. flava membrane lipid with the age of the bacterial culture. J. Gen. Microbiol. 65, 115, 1971.

BIBLIOGRAPHY

BIBLIOGRAPHY

Aasen, A.J., Francis, G.W., Jensen, S.L. Acta. Chem. Scand. 23, 2608, 1969.

Albro, P.W., Dittmer, J.C. Biochem. 8, 394, 1969.

Arpin, N., Francis, G.W., Jensen, S.L., Enzell, C. Acta. Chem. Scand. (In press).

Avigan, J., Goodman, D.S., Steinberg, D. J. Lipid Res. 4, 100, 1963.

Baird-Parker, A.C., Woodroffe, R.C.S. In "Progress in Microbiological Techniques" Butterworths (London). p. 85, 1967.

Bamji, M.S., Krinsky, N.I. Biochim. Biophys. Acta. 115, 276, 1966

Baumann, A.J., Simmonds, P.G. J. Bact. 98, 528, 1969.

Baxter, R.M. Can. J. Microbiol. 6, 417, 1960.

Bayer, M.E. J. Gen. Microbiol. 53, 395, 1968a.

_____ J. Virol. 2, 346, 1968b.

Bayley, S.T. J. Mol. Biol. 15, 420, 1966.

Bishop, D.G., Rutberg, L., Samuelsson, B. Eur. J. Biochem. 2, 454, 1967a.

_____ Eur. J. Biochem. 2, 448, 1967b.

Brown, A.D., Cho, K.Y. J. Gen. Microbiol. 62, 267, 1970.

Burchfield, H.P., Storrs, E.E. In "Biochemical Applications of Gas Chromatography" Academic Press (New York & London). p. 549, 1962.

Butler, T.F., Smith, G.L., Grula, E.A. Can. J. Microbiol. 13, 1471, 1967.

Canale-Parola, E., Borasky, R., Wolfe, R.S. J. Bact. 81, 311, 1961.

Chapman, G.B. J. Biophys. Biochem. Cytol. 6, 221, 1959.

_____ J. Bact. 79, 132, 1960.

Chapman, G.B., Hillier, J. J. Bact. 66, 362, 1953.

Cheesman, D.F., Lee, D.F., Zagalsky, P.F. Biol. Revs. 42, 131, 1967.

Cherny, N.E. Izv. Akad. Nauk. S.S.S.R. Ser. Biol. 2, 302, 1967.

Cho, K.Y., Salton, M.R.J. Biochim. Biophys. Acta. 116, 73, 1966.

Christian, J.H.B., Waltho, J.A. Biochim. Biophys. Acta. 65, 506, 1962.

Cohen, M., Panos, C. Biochem. 5, 2385, 1966.

Cole, R.M., Popkin, T.J., Boylan, R.J., Mendelson, N.M. J. Bact. 103, 793, 1970.

Colombo, P., Corbetta, D., Pirotta, A., Ruffini, G., Sartori, A. J. Chromatog. 3, 343, 1960.

Daems, W. Th., Persijn, J.-P. J. Roy. Micros. Soc. 81, 199, 1963.

Daron, H. J. Bact. 101, 145, 1970.

Edmonds, P., Cooney, J.J. J. Bact. 98, 16, 1969.

Edwards, M., Stevens, R.W. J. Bact. 86, 414, 1963.

Exterkate, F.A., Vrensen, G.F.J., Veerkamp, J.H. Biochim. Biophys. Acta. 219, 141, 1970.

Fiske, C.H., Subbarow, Y. J. Biol. Chem. 66, 375, 1925.

Fitz-James, P.C. J. Bact. 87, 1202, 1964.

Folch, J., Lees, M., Sloane-Stanley, G.H. J. Biol. Chem. 226, 497, 1957.

Francis, G.W., Hertzberg, S., Andersen, K., Jensen, S.L. Phytochem. 9, 629, 1970.

Freeman, J.A., Spurlock, B.O. J. Cell. Biol. 13, 437, 1962.

Friedberg, I., Avigad, G. J. Bact. 96, 544, 1968.

Gaylor, J.L., Delwiche, C.V. Anal. Biochem. 28, 361, 1969.

Ghosh, B.K., Carroll, K.K. J. Bact. 95, 688, 1968.

Ghosh, B.K., Murray, R.G.E. J. Bact. 93, 411, 1967.

Gilby, A.R., Few, A.V., McQuillen, K. Biochim. Biophys. Acta. 29, 21, 1958.

Glauert, A., Brieger, E.M., Allen, J.M. Exp. Cell. Res. 22, 73, 1961.

Glauert, A.M., Glauert, R.H. J. Biophys. Biochem. Cytol. 4, 191, 1958.

Glauert, A.M., Hopwood, D.A. Proc. Eur. Reg. Conf. El. Micros. 2, 759, 1961.

Glauert, A.M., Hopwood, D.A. J. Biophys. Biochem. Cytol. 7, 479, 1960.

Goodwin, T.W. In "Carotenoids" New York Chemical Publishing Co. p. 118, 1954.

Gordon, G.B., Miller, L.R., Bensch, K.G. Exp. Cell. Res. 31, 440, 1963.

Gough, T.A., Walker, E.A. J. Chromatog. 45, 14, 1969.

Grula, E.A., Butler, T.F., King, R.D., Smith, G.L. Can. J. Microbiol. 13, 1499, 1969.

Haidak, D.J., Mathews, C.K., Sweeney, B.M. Science. 152, 212, 1966.

Hammond, R.K., White, D.C. J. Bact. 103, 191, 1970.

Hard, G.C. J. Bact. 97, 1480, 1969.

Hertzberg, S., Jensen, S.L. Acta. Chem. Scand. 21, 15, 1967.

_____ Phytochem. 8, 1259, 1969a.

_____ Phytochem. 8, 1281, 1969b.

Higgins, M.L., Shockman, G.D. J. Bact. 101, 643, 1970.

Highton, P.J. J. Ultrastruct. Res. 26, 130, 1969.

_____ J. Ultrastruct. Res. 31, 247, 1970a.

_____ J. Ultrastruct. Res. 31, 260, 1970b.

Holman, C. Honours Thesis, University of St. Andre 1968.

Holt, S.C., Canale-Parola, E. J. Bact. 93, 399, 1967.

Hughes, R.C., Tanner, P.J., Stokes, E. Biochem. J. 120, 159, 1970.

Huston, C.K., Albro, P.W. J. Bact. 88, 425, 1964.

Huxley, H.E., Zubay, G. J. Biophys. Biochem. Cytol. 11, 273, 1961.

Jacob, F., Brenner, S., Cuzin, F. Cold Spr. Harb. Symp. Quant. Biol. 28, 329, 1963.

Jacob, F., Ryter, A., Cuzin, F. Proc. Roy. Soc. (Lond.) B164, 267, 1966.

Jensen, S.L. Ph.D. Thesis, I. Kommisjon Hos, Trondheim, 1962.

_____ J. Pure Appl. Chem. (In Press).

Jensen, S.L., Weeks, O.B., Strang, R.H.C., Thirkell, D. Nature 214, 379, 1967.

Kaneda, T. J. Biol. Chem. 238, 1222, 1963.

_____ Can. J. Microbiol. 12, 501, 1966.

_____ J. Bact. 93, 894, 1967.

_____ J. Bact. 95, 2210, 1968.

Kates, M. Adv. Lipid Res. 2, 17, 1964.

Kates, M., Hagen, P.O. Can. J. Microbiol. 42, 481, 1964.

Kellenberger, E., Ryter, A. In "Modern Developments in Electron Microscopy" Academic Press (New York & London), p. 335, 1964.

Kellenberger, E., Ryter, A., Sechaud, J. J. Biophys. Biochem. Cytol. 4, 671, 1958.

Kelly, M., Jensen, S.L. Acta. Chem. Scand. 21, 2578, 1967.

Kelly, M., Norgard, S., Jensen, S.L. Acta. Chem. Scand. 24, 2169, 1970.

Knivett, V.A., Cullen, J. Biochem. J. 103, 299, 1967.

Kuhn, R., Sorensen, N.A. Ber. Dt. Chem. Ges. 71, 1879, 1938.

Kushner, D.J., Onishi, H. J. Bact. 91, 653, 1966.

Landman, O., Ryter, A., Frehel, C. J. Bact. 96, 2154, 1968.

Larsen, H. In "Advances in Microbiol. Physiology" Academic Press (New York & London), 1, 97, 1967.

Ledbetter, M.C., Porter, K.R. J. Cell. Biol. 19, 239, 1963.

Lederer, E. Bull. Soc. Chim. Biol. 20, 611, 1938.

Lewis, V.J., Weaver, R.E., Hollis, D.G. J. Bact. 96, 1, 1968.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. J. Biol. Chem. 193, 265, 1951.

Luft, J.H. J. Biophys. Biochem. Cytol. 2, 799, 1956.

McCormick, A., Jensen, S.L. Acta. Chem. Scand. 20, 1989, 1966.

MacFarlane, M.G. Biochem. J. 79, 4P, 1961.

Mannozzi, V. J. Roy. Micros. Soc. 81, 141, 1963.

Mannozzi, V., Gautier, A. J. Ultrastruct. Res. 7, 436, 1962.

Marr, A.G., Ingraham, J.L. J. Bact. 84, 1260, 1962.

Mathews, M.M. Arch. fur Mikrobiol. 35, 139, 1960.

_____ J. Bact. 85, 322, 1963.

Mathews, M.M., Sistrom, W.R. J. Bact. 78, 778, 1959a.

Mathews, M.M., Sistrom, W.R. Nature, 184, 1892, 1959b.

Metcalfe, L.D., Schmitz, A.A. Anal. Chem. 33, 363, 1961.

Millonig, G. J. Appl. Physiol. 32, 1637, 1961.

Mirsky, R. Biochem. 8, 1164, 1969.

Mizushima, S., Ishida, M., Miura, T. J. Biochem. 81, 146, 1966.

Moore, S., Stein, W.H. J. Biol. Chem. 211, 907, 1954.

Morton, R.A. In "Absorption Spectra of Vitamins & Hormones" A. Hilger (London), 1942.

Moss, C.W., Dowell, W.R., Farshtchi, D., Raines, L.J., Cherry, W.B. J. Bact. 97, 561, 1969.

Moss, C.W., Dowell, V.R., Lewis, V.J., Schekter, M.A. J. Bact. 94, 1300, 1967.

Mower, H.F. Private communication. 1970.

Nandy, S.C., Sen, S.N. Ind. J. Exp. Biol. 5, 146, 1967.

Nanninga, N. Proc. Bur. Reg. Conf. El. Micros. Tipografia Poliglotta Vaticana (Rome), p. 443, 1968.

Neale, E.K., Chapman, G.B. J. Bact. 104, 518, 1970.

Neuberger, A., Gottschalk, A., Marshall, R.D. In "Glycoproteins Elsevier Publishing Co. (Amsterdam, New York & London), p. 273, 1966.

Norgard, S., Francis, C.W., Jensen, A., Jensen, S.L. Acta. Chem. Scand. 24, 1460, 1970.

O'Leary, W.M. Bact. Revs. 26, 421, 1962.

Palade, G.E. J. Exp. Med. 95, 285, 1952.

Petitprez, A., Roos, P., Tacquet, A. J. Micros. 6, 229, 1967.

Petracek, F.J., Zechmeister, L. Anal. Chem. 28, 1484, 1956.

Petter, H.R.M. Proc. Koninkl. Akad. Wetenschap. Amsterdam. 34, 1417, 1931.

Prebble, J. J. Gen. Microbiol. 52, 15, 1968.

Pridham, J.B. Apal. Chem. 28, 1967, 1956.

Pontefract, R.D., Bergeron, G., Thatcher, F.S. J. Bact. 97, 367, 1969.

Pontefract, R.D., Thatcher, F.S. J. Ultrastruct. Res. 30, 78, 1970.

Randerath, K. "Thin-layer Chromatography" Academic Press (New York & London) 1964.

Razin, S., Morowitz, H.J., Terry, T.M. Proc. Nat. Acad. Sci. 54, 219, 1965.

Remsen, C.C. Arch. fur Mikrobiol. 61, 40, 1968.

Reynolds, E.S. J. Cell. Biol. 17, 208, 1963.

Roth, M.M. J. Bact. 93, 506, 1967.

Roth, M.M., Krinsky, N.I. Biochim. Biophys. Acta. 203, 357, 1970.

Rottem, S., Razin, S. J. Bact. 92, 714, 1966.

Ryter, A. Folia Microbiol. (Prague), 12, 283, 1967.

_____ In "Current Topics in Microbiology & Immunity" Springer Verlag (Berlin), 49, 451, 1969.

Ryter, A., Jacob, F. Prot. Biol. Fluids. 15, 267, 1967.

Ryter, A., Kellenberger, E. Z. Naturforsch. 13, 597, 1958.

Ryter, A., Landman, O. J. Bact. 88, 457, 1964.

_____ In "Microbial Protoplasts, Spheroplasts & L-forms" Williams & Wilkinson Co. (Baltimore), p. 110, 1967.

Sabatini, D.D., Bensch, K., Barrnett, R.J. J. Cell. Biol. 17, 19, 1963.

Sabatini, D.D., Miller, F., Barrnett, R.J. J. Hist. Cyt. 12, 57, 1964.

Salton, M.R.J. Trans. N.Y. Acad. Sci. 29, 764, 1967a.

_____ In "The Specificity of Cell Surfaces" Prentice Hall Inc. (New Jersey), p. 71, 1967b.

_____ Prot. Biol. Fluids. 15, 279, 1967c.

_____ Ann. Rev. Microbiol. 21, 417, 1967d.

Salton, M.R.J., Ehtisham-ud-din, A.F.M. Austr. J. Exp. Biol. Med. Sci. 43, 255, 1965a.

Salton, M.R.J., Freer, J.H. Biochim. Biophys. Acta. 107, 531, 1965b.

Salton, M.R.J., Freer, J.H., Ellar, D.J. Biochem. Biophys. Res. Comm. 33, 909, 1968.

Salton, M.R.J., Netschey, A. Biochim. Biophys. Acta. 107, 539, 1965c.

Saperstein, S., Starr, M.P. Biochim. Biophys. Acta. 16, 482, 1955.

Schneider, W.C. In "Methods in Enzymology" Academic Press (New York & London), 3, 680, 1957.

Scott, T.A., Melvin, E.H. Anal. Chem. 25, 1656, 1953.

Shockman, G.D., Kolb, J.J., Bakay, B., Conover, M.J., Toennies, G. J. Bact. 85, 168, 1963.

Smith, P.F. J. Gen. Microbiol. 32, 307, 1963.

_____ Lipids, 4, 331, 1969.

Smith, P.F., Koostra, L., Mayberry, W.R. J. Bact. 100, 1166, 1969.

Sperry, W.M., Webb, M. J. Biol. Chem. 187, 97, 1950.

Stephens, W.L., Starr, M.P. J. Bact. 86, 1070, 1963.

Steensland, H., Larsen, H. J. Gen. Microbiol. 55, 325, 1969.

Strang, R.H.C. Ph.D. Thesis, University of St. Andrews, 1968.

Strang, R.H.C., Thirkell, D. Phytochem. 8, 431, 1969.

Subbarayan, C., Cama, H.R. Ind. J. Biochem. 3, 225, 1966.

Suganuma, A. J. Cell. Biol. 30, 208, 1966.

_____ J. El. Micros. 17, 315, 1968.

Sweeley, C.C., Bentley, R., Makita, M., Wells, W.W. J. Am. Chem. Soc. 85, 2497, 1963.

Takeda, Y., Ohta, T. Hoppe-Seylers Z. 268, 1, 1941.

Thirkell, D. J. Gen. Microbiol. 55, 337, 1969.

Thirkell, D., Strang, R.H.C. J. Gen. Microbiol. 49, 53, 1967.

Thirkell, D., Strang, R.H.C., Chapman, J.R. J. Gen. Microbiol. 49, 444, 1967.

Thomas, T.D., Lyttleton, P., Williamson, K.I., Batt, R.D. J. Gen. Microbiol. 58, 381, 1969.

Thornley, M.J., Horne, R.W., Glauert, A.M. Arch. fur Mikrobiol. 51, 261, 1965.

Tomasz, A., Jamieson, J.D., Ottolenghi, E. J. Cell. Biol. 22, 453, 1964.

Tornabene, T.G., Bennett, E.O., Oro, J. J. Bact. 94, 344, 1967.

Vorbeck, M.L., Marinetti, G.V. Biochem. 4, 296, 1965.

Ward, J.B., Perkins, H.R. Biochem. J. 106, 391, 1968.

Weeks, O.B., Andrewes, A.G. Arch. Biochem. Biophys. 137, 284, 1970.

Weeks, O.B., Andrewes, A.G., Brown, B.O., Weedon, B.C.L. Nature, 224, 879, 1969.

Weeks, O.B., Garner, R.J. Arch. Biochem. Biophys. 121, 35, 1967.

Whistler, R.L., Wolfrom, M.L. "Methods in Carbohydrate Chemistry" Academic Press (New York & London), 1, 388, 1962.

White, D.C., Frerman, F.E. J. Bact. 95, 2198, 1968.

Widra, A. J. Bact. 78, 664, 1959.

Wolken, J.J., Schwertz, F.A. Nature, 177, 136, 1956.

Yamaguchi, T., Tsukakoshi, N., Tamura, G., Arima, K. Agr. Biol. Chem. 31, 475, 1967.

Yudkin, M.D. Biochem. J. 98, 923, 1966.